ALLELOPATHIC IMPACT OF CALOTROPIS ROCERA (AIT.) R. BR. ON SOME SELECTED WEEDS AND CROPS

Submitted For the Award of Degree of

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By

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This is to certify that the thesis entitled **"Allelopathic impact of *Calotropis procera* (Ait.) R. Br. on some selected weeds and crops"** embodies a faithful record of bonafide research work carried out by **Ms. Aasifa Gulzar** at the Department of Botany, Aligarh Muslim University, Aligarh under my guidance and supervision. Any part of it has not been submitted for the award of any other degree or diploma. She is allowed to submit her thesis to the Aligarh Muslim University, Aligarh for the consideration of the award of the degree of **Doctor of Philosophy** in **Botany**.

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ABSTRACT

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Weeds cause a reduction in crop yield. They cause crop productivity loss on an average of 34%. They act as a shield for the crop plants for available nutrients, space, light and moisture. Hence, in the presence of weeds physiological activities and growth of crops are negatively affected. In addition, they deteriorate crop quality, clog waterways, cause health problems in humans and look unsightly in amenity areas such as garden, parks, pathways and pavements, etc. Weeds also cause fire hazards, besides being determinable to crop yields and unappealing. Weeds also are the permanent hosts of insects and pathogens, adding more complications to their control. The allelopathic natures of about 240 weed species are reported and interferes with the growth and production of crops. Hence, since the ancient times, weeds have been documented as serious plant pests. Although there are several methods to control weeds viz. mechanical, physical, biological, chemical methods and herbicide use but each are accompanied with negative effects, make it to find necessary to diversify or other weed management options. The use of allelopathic behavior is one of the new options for sustainable weed management. Where allelopathy is, direct influence of chemicals released from one plant in environment and then influence on the growth and development of another, may provide an alternative to promising weed control methods. This is suggesting that allelopathy is directly or indirectly involved in weed management during crop production. In this regard, allelopathic plants and their products for managing weeds in a sustainable manner has been focused with much attention. Allelochemicals released from the allelopathic plants replace the use of synthetic herbicides for weed management and therefore, cause less pollution, safer agricultural products as well as alleviate human health concerns. Suppressing weeds by harnessing the allelopathic phenomenon is included among the important innovative weed control methods. Allelopathic weed control may be applied as a single strategy in certain cropping systems, such as organic farming. Further, it can be combined with other methods to achieve integrated weed management. Therefore, for the management of agricultural weeds, it is worthwhile to explore the strong allelopathic activity of the plant.

The study suggests that *Calotropis procera* (Ait.) R. Br. is an allelopathic plant, which is capable of suppressing the germination and growth of various test species (crops and weeds). *C. procera* commonly known as ‘Aakawa’ belonging to the family Asclepiadaceae, is an erect perennial shrub whose members are distributed

throughout the world in tropical and sub-tropical regions. With wide ecological distribution in many regions of Aligarh district of Uttar Pradesh, India, it mainly shows its common occurrence and invasion around the agricultural lands and farms. Its widespread and persistent occurrence near barley, oat, rice, sorghum, maize, cotton, sugarcane fields and especially around wheat crop fields makes it suspicious to cause some adverse effect on these crops through allelopathic interaction. Generally, the plant contains the allelochemicals that plays an important role in the formation of natural habitats and to compete with other species. For increasing organic materials in agroecosystems, it is recycled as a green manure, where it may change communities and inhibit crop growth and production. The successful invasion of *C. procera* can be attributed due to its continuous flowering and autogamy in invading areas, high seed production, efficiently dispersed by wind and fast growth after establishment. It was visually observed that any field left fallow is likely to be invaded by this weed and thus affecting the growth of other plants. The reason for this impact of weed in the area it invades cannot be ascertained, in the absence of any study, it is hypothesized that the success of the invasive tendencies of the weed are due to its allelopathic properties.

In my allelopathic studies, aqueous extracts and organic extracts, rhizosphere soil, residues and root residues of *C. procera* in various experiments, invariably reduced the germination, plumule growth, radicle growth, dry weight and alter chromosome morphology of their respective recipient species [weeds (*Cassia tora* L., *Cassia sophera* L., *Chenopodium album* L., *Cannabis sativa* L.) and crops (*Pisum sativum* L., *Triticum aestivum* L., *Brassica oleraceae* var. *botrytis* L., *Spinacia oleracee* L. and *Allium cepa* L.)].

PHYTOTOXINS IN RHIZOSPHERE INDUCE ALLELOPATHIC POTENTIAL WITH RESULTS DISCUSSED BELOW

In the present study, various biological and ecological features of *C. procera* at vegetative and flowering stage was studied. It can be seen that growth parameters of weed (*C. procera*) such as average length of aboveground and belowground parts, number of leaves/plant, secondary roots/root and fresh and dry biomass of different parts both at vegetative as well as flowering stage dictates its fast growth. Further, the rhizosphere area increases nearly three times from vegetative to flowering stage. The species flower and fruit in all seasons. It has special seed dispersion mechanism

(anemophily) and seeds remain viable for a long period. The seeds are carried from one place to another in mud or sand which man use for many kind of constructions. Finally, the denudation of local vegetation also affords an apportunity to the plant to propagate under a reduced strain of competition to which it is subjected and thus aggressive stands of *C. procera* are commonly met within their places of occurrence.

Some weed (*C. tora*, *C. sophera*, *C. album* and *C. sativa*) and crop (*P. sativum*, *T. aestivum*, *B. oleraceae* var. *botrytis* and *S. oleracea*) plants were studied under the rhizosphere soil, however the seedling growth and dry weight was decreased compared to control soil. The soil collected from *C. procera* infested areas (both at vegetative and flowering stage) as well as from this weed-free (control) area were analyzed for some physico-chemical characteristics apart from the amount of macro and micronutrients. A significant difference was observed in the amount of various elements in *C. procera* at vegetative and flowering stages. In general, the soils were slightly alkaline, both in control as well as *C. procera* infested area and a little and statistically significant difference observed among these. pH, electrical conductivity, phenolic contents, organic carbon and organic matter were also studied in infested soil as well as control soil. Electrical conductivity was found to be maximum in *C. procera* infested soil at flowering stage followed by vegetative stage and control soil. The percent organic carbon and organic matter was found to be maximum in soil supporting *C. procera* plants at vegetative stage followed by soil at flowering stage and control. Likewise, the amount of Na and Mg followed the same trend. Although the amount was more in case of chlorides, bicarbonates and calcium in contrast to control soil at vegetative and flowering stage, yet decrease observed in soil with plants at vegetative stage compared to those at flowering stage. In case of micronutrients like Fe, Zn, Mn and Cu, a similar trend was observed as in case of N, P and K, i.e. the maximum amount of respective element or nutrient was found in soil at flowering stage, followed by vegetative stage and control. In addition, EDX-SEM analysis also detected both macro and micronutrients (O, Mg, Al, Si, K, Ca, Fe, Zn, N, Mn, Cu, Cl and P.) in the rhizosphere soil of *C. procera*. Thus, it becomes clear from the study that soil supporting *C. procera* invaded plants either at vegetative and flowering stage is not deficient in any of nutrients rather the status of both macro-and micro-nutrients are better in comparison to control favoring the better growth of *C. procera*. Besides, macro and micronutrients, the amount of total phenolics was also

estimated in the soils. It was found to be maximum in soil at vegetative stage followed by that in soil with flowering stage and least in control soil. Upon HPLC analysis of rhizosphere soil four phenolic acids (ferulic acid, vanillic acid, *p*-coumaric acid and benzoic acid) were identified. The studies indicate that phenolics are present in the rhizosphere soil of *C. procera* that adversely affects the early growth of test plants compared to control.

RESULTS OF ALLELOPATHIC IMPACT OF AQUEOUS EXTRACT ON RECIPIENT SPECIES ARE AS FOLLOWS

Laboratory experiments was conducted to determine the effects of aqueous extracts of different parts (root, stem and leaves) of *C. procera* (at mature stage) on different crops, i.e *T. aestivum*, *B. olerace* var. *botrytis*, *S. oleracea* , *P. sativum* and weed plant, i.e. *C. sophera*, *C. tora*, *C. album* and *C. sativa*. Generally, aqueous extracts of different parts had significant retardatory effect on radicle, plumule length and dry biomass of all test plant. In all these cases, retardatory effect was increased with increasing concentration of aqueous extracts, i.e. 0.5 to 4%.

The phytotoxic effect of leaf extract on the radicle length, plumule length and dry biomass of all these test plants was greater than that of root extract. However, stem extract shown least retardatory effect on all these test plants. Generally, in studies with an aqueous extract, the observed retardatory effects may be attributed to changes in pH and osmotic potential. In the present study, pH of extracts ranged from 6.66 to 6.95, likewise, the osmotic potential ranged from -0.046 to -0.096 bars was observed and an appreciable amount of phenolics was determined in all the extract and their amount increased with increasing extract concentration which were maximum in leaf extract. In addition to the reduction in seedling growth and dry biomass, the treated seedling of *P. sativum* also shows visual symptoms in the form of darkening and rottening, with the increased number of seminal roots, reduced lateral root production, decreased extension of root and root tips swallowed (club-like appearance). The *Cassia* seedling also pronounced negative geotropism at 4% concentration of leaf aqueous extract. The SEM studies revealed that leaf aqueous treatment caused foliar ultramorphological changes of *Cassia* leaf epidermal surface when compared with the control. The SEM analysis of the leaf surface revealed disruption of epidermal cells in the form of canals and formation of cyst like structures instead of being smooth as depicted in the control treatment.

THE RESULTS OF ALLELOPATHIC EFFECT OF RESIDUE AMENDED SOILS (RS AND RES) AND RESIDUE EXTRACTS (RE) ARE AS FOLLOWS

The present study investigates the effect of residue of *C. procera* in soil as well as under laboratory conditions. Soils were infested with different amounts of *Calotropis* residue to determine the change in soil chemistry, phenolic content and the phytotoxic effects on crops, i.e. *S. oleracea, B. oleracea* var. *botrytis* and weeds, i.e. *C. sativa* and *C. album.* Root length, shoot length and dry biomass were significantly decreased with increasing concentration of residues amended soil (RS), residue extract amended soil (RES) and aqueous extract of residues (RE). Specific studies conducted to find out the nutrient status in amended soils as well as the residue itself and determination of pH and electrical conductivity of extracts. The pH of aqueous extracts (RE) was near neutral with increasing concentration. Further, the phenolic content was maximum at highest concentration of aqueous extracts of residue (RE). In case of amended soils, the differences in physico-chemical properties were apparent. However, the differences in pH were not very sharp, the electrical conductivity was enhanced several times, in both RS and RES. Likewise, significant increase in organic matter was also seen. A sharp increase in available N, P and K was observed and in each case, the increase was more in RS compared to RES. Besides macronutrients, the amount of micronutrients like Zn, Mn, Fe and ions like Cl and HCO_3 also increased in amended soil compared to unamended soil. The amount of Cu, however, did not show much change in both cases (amended and unamended). Besides, the presence of phenolics in amended soils, the amount was also detected.

ROOT RESIDUE AMENDED SOILS (RRS AND RRES) AND ROOT RESIDUE EXTRACTS (RRE) WITH RESULTS DISCUSSED AS FOLLOWS

A study was conducted to explore the allelopathic potential of *C. procera* root residue to determine its possible interactions with soil nutrient status and characteristics features of *C. procera* roots by quadrate. Crops, i.e *S. oleracea, B. oleracea* var. *botrytis* and weeds, i.e. *C. sativa* and *C. album* showed phytotoxic effect against root residue amended soils (RRS), root residue extract amended soil (RRES) and aqueous extract of root residue (RRE). Shoot length, root length and dry biomass was reduced in response to RRS, RRES and RRE in contrast to control. In order to check the possible interaction of phenolics with soil nutrients and other soil particles, specific studies were also undertaken in this regard. Not much change in soil pH was

observed. Further, the phenolic content was maximum at highest concentration of aqueous extracts of root residue (RRE). However, with the amendment of root residues at 2 and 4% the soil was more alkaline, i.e. pH increased towards alkalinity. Similarly, organic matter also measured to be more in the amended soils compared to control. Further, the contents of macro and micronutrients were estimated to increase with increasing the concentration in all the amended soils compared to control. In all parameters, the rate of increase was more in case of soils amended with root residue compared to soil amended with root extracts. However, the elemental analysis of root residue was also determined. The soils were nutrient rich, with an increased EC, indicating greater nutrient availability and thus enriching of the RRS and RRES. Moreover, phytotoxicity and quantification of phenolics from the RRS and RRES in pure form against test plants indicates their direct involvement in the observed growth reduction.

THE RESULTS OF EXTRACTED ALLELOCHEMICALS BY AQUEOUS AND ORGANIC SOLVENTS ON GERMINATION BEHAVIOR AND PHYSIOLOGICAL PARAMETERS ARE AS FOLLOWS

Varieties of ways were employed to extract the allelochemicals from *C. procera* fresh or from dried powder. Freshly prepared allelochemicals represented the glycosidic forms of secondary metabolites or phenolics. These were extracted in water in the form of aqueous leachates. The glycosidic bonds in the aqueous leachates were cleaved by acid hydrolysis and thus aglyconic forms separated from the dried powder, allelochemicals were extracted in different solvent system with decreasing polarity.

For the bio-efficacy studies, parameters of carbohydrate content, protein content and chlorophyll content were assessed through standardized methods. Germination parameters, i.e. percentage, seed vigour, radicle length and plumule length were employed to test the effect of *C. procera* allelochemicals. Both physiological and germination parameters were conducted on crops, i.e. *T. aestivum*, *S. oleracea* and weeds, i.e. *C. sativa* and *C. album.* One thing is very clear from the result of this experiment that *C. procera* leaves' allelopathic exert a very negative influence on the acid soluble and water-soluble carbohydrates. It is very well depicted by an increased amount of carbohydrates points out to the fact that the plant is under stress. As compared to the control, chlorophyll content in crops and weeds decreased dramatically and the aqueous leachates proved to be much more effective than the other treatment in this case too. Similarly, it was observed that the plant protein

content was found to be reduced in all the treatments as compared to control. However, significant inhibition of germination dynamics and seedling growth of crops and weeds was noticed under the influence of aqueous and organic fraction solvents. Differential level of phytotoxicity in response to aqueous extracts and organic fractions exhibited against test species might occured due to the variable chemical nature of the compounds used for extraction.

THE RESULTS OF HPLC ANALYSIS OF ALLELOCHEMICALS ARE AS FOLLOWS

C. procera reduced the germination and seedling growth of test species by its allelochemicals that were identified to be the water soluble phenolic acids. The study conducted involved the extraction and identification of phenolic acids from methanolic extracts of leaf, stem and root by High Performance Liquid Chromatography (HPLC). Nine phenolic acids were found to be present in organic extracts of different parts of *C. procera*. In the green leaves, these were caffeic acid, gentistic acid, catechol, gallic acid, syringic acid, ellagic acid, resorcinol, *p*-coumaric acid and *p*-hydroxy benzoic acid with different retention time and quantities. Although eight phenolic acids (vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, syringic acid, gallic acid, pyrogallic acid and *p*-coumaric acid) detected in stem and root extract were similar, however they show difference in terms of retention time and quantities that reflect their differential pytotoxicity. However, the furoic acid in stem extract and ferulic acid in root extract were identified different ones with their respective retention time and quantities in addition to these eight phenolic acids. The presence of phenolic acids in the different parts of *C. procera* indicates that these play an important role in imparting phytotoxic/allelopathic property to this weed.

THE RESULTS OF SEM ANALYSIS AND LEAF DIPPING EXPERIMENT ARE AS FOLLOWS

The SEM analysis depicts the presence of numerous sessile non-glandular trichomes and stomata on leaf surface of adaxial and abaxial surfaces. A dipping experiment involving dichloromethane and distilled water was used to determine the solubility of the contents of trichomes and ultramorphological changes in trichomes found on the leaf surface. The inhibitory substances present in trichomes are represented by soaking the leaves of *C. procera* in different concentrations (25%,50%,75% and 100%) of organic solvent dichloromethane and distilled water at

different time intervals (five seconds, ten seconds and 24 hours). From the results, it was stated that organic solvent infusion did not exhibit typical hormesis graph compared to distilled water infusion. Besides the SEM analysis of *Calotropis* leaves soaked in dichloromethane depicted alteration in trichome structure, stomata and epidermal surface. Positive results proved that trichomes are possible sources of allelochemicals on both adaxial and abaxial surfaces of young and mature leaves. Structures found on the leaves of the plant could possibly contain the allelochemicals used by the plant to ensure its successful invasion growth. Results also concurred that the allelochemicals causing allelopathic potential of *C. procera* are probably water-soluble (polar compounds).

THE RESULTS OF ALLELOTOXICITY OF LEAF AQUEOUS EXTRACT ON CYTOMORPHOLOGY ARE AS FOLLOWS

The study was conducted to reveal the allelopathic effect of leaf aqueous extract on chromosomal abnormalities, mitotic index (MI), frequency of chromosomal abnormalities and variation in the shape of *A. cepa* meristematic root tip cells. *Allium* root tips were exposed to different concentrations (0.5%,1%,2% and 4%) of leaf aqueous extract and distilled water as control. Various types of chromosomal abnormalities (stickiness, delayed mitosis, disturbed phases, micronuclear formation, bridges, lagging chromosones, C-mitosis and U-mitosis) were induced upon exposure to different concentrations of leaf aqueous extract compared to control. All four stages of mitosis (prophase, metaphase, anaphase and telophase) were affected with different abnormalities in chromosome structure and shape. The mitotic index decreased in all the treated samples very significantly. The result revealed that the frequency of dividing cells reduced remarkably in the treated samples than the control ones. The prophase exhibit abnormalities in the form of despirilization of chromosomes along with the formation of micronucleus. Many chromosomal abnormalities were encountered in metaphase and anaphase stage of *Allium* root tip cells. They exhibited many chromosomal abnormalities in metaphase such as disturbed metaphase, sticky metaphase, star metaphase, ball metaphase, C-metaphase and U-metaphase. Abnormalities detected in anaphase were sticky anaphase, disturbed anaphase, anaphase with double bridge, multipolar anaphase, early anaphase, disturbed multipolar anaphase, sloping anaphase and delayed anaphase. Leaf extract treated cells of *A. cepa* showed variation in the shape of cells along with the dislocation of

nucleus upon exposure to 0.5%,1%,2% and 4% treatment in contrast to control. Hence keeping in view these earlier reports and the results of this study *Calotropis* can be exploited for bioherbicides.

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LIST OF ABBREVIATIONS

µg	Microgram	**WF**	Water fraction
µS	Microsecond	**DTPA**	Diethylenetriaminepentaacetic acid
AAMPO	2-amino-7- methoxy-3H-phenoxazin-3-one	**EDTA**	Ethylenediamine Tetra Acetic Acid
AMPO	2-amino-7- methoxy-3H-phenoxazin-3-on	**DMSO**	Di-methyl Sulphoxide
ANOVA	Analysis of variance	**µg**	Microgram
APO	2-aminophenoxazin-3-one	**AOAC**	Association of Official Analytical Chemists
BOA	2-benzoxazolinone	**w/v**	Weight by volume
cm	Centimeter	**ml**	Mililitre
CRETS	Continuous Root ExudatesTrapping system	**SPME**	Solid Phase Microextraction
DAS	Days after sowing	**nm**	Nanometer
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	**MF**	Methanolic fraction
ECAM	Equal Compartment AgarMedium	**WSF**	Water soluble fraction
GA	Gibberllic acid	**ASF**	Acid Soluble fraction
HCl	Hydrochloric acid	**GC–MS**	Gas chromatography- mass spectrometer
HPLC	High Performance Liquid Chromatography	**ASF**	Acid soluble fraction
IAA	Indole 3-acetic acid	**OP**	Osmotic potential
LSD	Least significant difference	**mg**	Milligram
MBOA	6-methoxy-benzoxazolin-2(3H)-one	**mg**	Milligram
MI	Mitotic index	**RS**	Residue amended soil
mm	Millimeter	**AAS**	Atomic AbsorptionSpectroscopy
mS	MilliSiemens	**v/v**	Volume by volume
OH	Hydroxyl	**NRCWS**	National Research for Weed Science
PF	Petroleum fraction	**RSIC**	Regional Sophisticated Instrumentation Centres

RE	Residue extract	**EC**	Electrical conductivity
RES	Residue extract amended soil	**US**	Unamended soil
RH	Relative humidity	**IARI**	Indian Agriculture Research Institute
rpm	Revolutions per minute	**RT**	Retention time
RRE	Root residue extract	**ISTA**	International seed testing association
RRES	Root residue extract amended soil	**SD**	Standard deviation
RRS	Root residue amended soil	**DMRT**	Duncan Multiple Range Test
SEI	Secondary Electron Image	**g**	Gram
SEM	Scanning Electron Microscopy	**CF**	Chloroform fraction
SPRE	Solid Phase Root Zone Extraction	**USIF**	University Sophisticated Instrument Facility
UV	Ultraviolet	**EDX-SEM**	Energy Dispersive X-ray Spectrometer Scanning Electron Microscopy

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Chapter-1

Introduction

INTRODUCTION

Weeds constantly compete with crop plants to cause a considerable loss in their productivity (Jabran *et al*., 2015). Crop productivity can be lowered by weeds on an average of 34% (Oerke, 2006). The potential yield reductions by weeds in some important crops are: wheat 23%, soybeans 37%, rice 37%, maize 40%, cotton 36%, and potatoes 30% (Oerke, 2006). They act as a shield for the crop plants for available nutrients, space, light and moisture (Gulzar *et al*., 2015a). Hence, in the presence of weeds physiological activities and growth of crops are negatively affected (Rajcan and Swanton, 2001). In addition, they deteriorate crop quality, clog waterways, cause health problems in humans and look unsightly in amenity areas such as garden, parks, pathways and pavements, etc (Singh *et al*., 2003c). Weeds also cause fire hazards, besides being determinable to crop yields and unappealing (Zimdahl, 1999; Singh *et al*., 2003c). Weeds also are the permanent hosts of insects and pathogens, adding more complications to their control (Singh *et al*., 2003c). Hence, since the ancient times, weeds have been documented as serious plant pests (Zimdahl, 2013). Weeds have always played a role throughout the domestication of crop plants, which necessitated practicing weed control measures (Oerke *et al*., 1999; Zimdahl, 2013). Among the ancient methods of weed control, pulling by hand, cutting and physically smothering weeds are widely used (Oerke *et al*., 1999; Young *et al*., 2014). Over time, hand tools were developed to till soils in order to control weeds. During recent times, herbicides and other modern means of weed control have been used. However, since the beginning of agriculture, hand weeding, mechanical weeding and herbicide applications have been most relied upon weed control methods (Griepentrog and Dedousis, 2010; Bergin, 2011; Rueda-Ayala *et al*., 2011; Chauvel *et al*., 2012; Jabran *et al*., 2015). These weed control methods have served to keep weed infestations low and improve the crop productivity throughout the world. Despite the significant contribution of these weed control methods in improving crop productivity, certain challenges are also associated with them. The challenges associated with conventional weed control methods (e.g., hand weeding, mechanical control, herbicides, etc.) make it imperative to develop diversity in the current weed control methods. Further, the costs of weed eradication are also enormous (Singh *et al*., 2003c). Pimentel *et al*. (2001) has estimated out that the loss in crop yield due to weeds in the U.S. is about 12% and it costs nearly US$35 billion to control them. As observed by Heady and

Child (1994) that in 1960,s in the United States, Department of Agriculture due to invasion of land by weeds and brush increased costs of management, estimated at U$ 250000000 annually on Western United States rangelands, followed by US$ 340000000 loss in 1989 in seventeen Western United States with concomitant plant poisoning and physical injury. In addition to the direct losses, approximately $4 billion is spent each year on herbicides used to control pest weeds (Inderjit, 2008). However, the costs are even more in developing countries (Singh *et al*., 2003c).

In light of these characteristics of weeds and their hazards, it becomes imperative to control them. Therefore, efforts are being made to find out alternative low-input strategies for weed management, although numbers of management practices are available. Where allelopathy is the direct influence of a chemicals released from one plant in the environment and then influence on the growth and development of another (Babula *et al*., 2009; Farooq *et al.,* 2011; Cheng and Cheng, 2015; El-Khatib *et al*., 2016). The allelopathic nature of about 240 weed species is reported and interfere with the growth and production of crops (Qasem and Foy, 2001; Singh *et al.*, 2003c). In this regard, allelopathic plants and their products for managing weeds in a sustainable manner has been focused with much attention (Sodaeizadeh *et al.,* 2010). Allelochemicals released from the allelopathic plants replace the use of synthetic herbicides for weed management and therefore, cause less pollution, safer agricultural products (Sodaeizadeh *et al.,* 2010) as well as alleviate human health concerns (Khanh *et al*., 2007). Suppressing weeds by harnessing the allelopathic phenomenon is included among the important innovative weed control methods (Jabran and Farooq, 2013; Zeng, 2014). Allelopathic weed control may be applied as a single strategy in certain cropping systems, such as organic farming. Further, it can be combined with other methods to achieve integrated weed management. Under allelopathic weed control, the allelopathic potential of crops are manipulated in such a way that the allelochemicals from these crops reduce weed competition. The living plants or their dead materials express the allelopathic activity through the exudation of allelochemicals. Allelopathic weed control can be implemented by growing allelopathic plants in close proximity to weeds, which promote production of these chemicals (Tesio and Ferrero, 2010), or by placing the allelopathic materials obtained from dead plants in close proximity to weeds. The decomposing plant material releases allelochemicals, which are absorbed by the target

weeds. The most important example for such cases includes the use of allelopathic plant residues for weed control (Tabaglio *et al.,* 2013). Allelopathic weed control can also be implemented by growing allelopathic plants in a field for a certain period of time, in order for their roots to exude allelochemicals. Crop rotation is the most important example for such allelopathic weed control (Farooq *et al*., 2011). Another way to control weeds through allelopathy includes obtaining allelochemicals in a liquid-solution by dipping the allelopathic chaff in water for a certain period of time. Several researchers have advocated using this way of weed control either alone or in combination with other methods of weed control (Jabran *et al*., 2010; Khan *et al*., 2012; Razzaq *et al*., 2010, 2012). Therefore, for the management of agricultural weeds, it is worthwhile to explore the strong allelopathic activity of the plant.

1.1. SELECTION OF ALLELOPATHIC PLANT CALOTROPIS PROCERA (Ait.) R. Br.

1.1.1. Identity and taxonomy

Kingdom Plantae

Class Magnoliopsida

Order Gentianales

Family Asclepiadaceae

Genus *Calotropis*

Species *procera*

Common name Milkweed, Aakawa

1.1.2. Description

Calotropis procera (Ait.) R. Br. commonly known as ‘Aakawa’ belonging to the family Asclepiadaceae, is an erect perennial shrub whose members are distributed throughout the world in tropical and sub-tropical regions. With wide ecological distribution in many regions of Aligarh district of Uttar Pradesh, India, it mainly shows its common occurrence and invasion around the agricultural lands and farms (Gulzar *et al*., 2015a). The map of Aligarh district chosen for an experimental purpose is shown in Plate 1. *C. procera* is a shrub or small tree 2-4 m tall (rarely up to 6 m tall), with distinctive grey-green waxy leaves (Csurhes, 2009). Various morphological characters of plants are represented in Plate 2. The stems are gray-green, smooth, somewhat crooked and covered with a soft, thick, corky bark (Grace, 2006, 2009; Csurhes, 2009). Exudation of milky, sticky sap occurs when the plant is broken, cut or

injured. Branches often arise from the base of the plant. Leaves are sessile, glaucous, ovate to obovate, 5-20 cm long and 4-10 cm wide, with six prominent veins on the underside and a short, pointed tip (apex) with the opposite arrangement along the stems. With the leaf base cordate (heart-shaped), they clasp the stem part. The plant is not deciduous. Dense, multi-flowered, umbellate cyme dictates its inflorescence. Globular flower buds are present. The open corolla is white and pink/purple and 2-3 cm in diameter. The flowers are grouped in umbels and each flower bears five petals. The fruit are choke-like, take the shape of gray-green pods, 8-12 cm long and contains hundreds of seeds, each with a tuft of long (2-3 cm) silky hairs at one end (Csurhes, 2009). Each seed weighs 6-7 mg (Amritphale *et al*., 1984). The roots can form large tubers and are up to 4 m long (Grace, 2006). Toxic nature is reported from all parts of the plant. The compound calotropin present in the milky sap affects the heart and causes blistering and irritation in people (Staples and Herbst, 2005; Csurhes, 2009). Seeds are the main source of reproduction, although suckers can be produced from the roots. Seeds of *C. procera* also germinate in cattle dung.

1.2. DISTRIBUTION IN ALIGARH

A survey of Aligarh district of Uttar Pradesh, India confirms that plant is mainly found on dry, sandy and alkaline soils, waste and fallow lands along roads, along roadsides, streets, residential colony parks, sand dunes as well as in crop fields as a weed.

1.3. INTERFERENCE AND ALLELOPATHIC NATURE OF C. PROCERA

C. procera was an important weed believed by 62% respondents, as per a survey of 10 experienced landholders and weed specialists in the Northern Territory (Grace, 2006; Csurhes, 2009). Similarly, 72% felt that government should invest in control (Csurhes, 2009). Besides, an average of $7625 was spent by surveying landholders controlling *C. procera*. In frequently disturbed areas, it occurs as a major to intermediate weed and occurs along the road verges throughout India (Sharma *et al*., 2010). Its widespread and persistent occurrence near barley, oat, rice, sorghum, maize, cotton, sugarcane fields and especially around wheat crop fields makes it suspicious to cause some adverse effect on these crops through allelopathic interaction (Yasin *et al*., 2012). Generally, the plant contains the allelochemicals that plays an important role in the formation of natural habitats and to compete with other species. For increasing organic materials in agroecosystems, it is recycled as a green

manure, where it may change communities and inhibit crop growth and production (Al-Zahrani and Al-Robai, 2007). The successful invasion of *C. procera* can be attributed due to its continuous flowering and autogamy in invading areas, high seed production, efficiently dispersed by wind and fast growth after establishment (Sharma *et al*., 2010; Leal *et al*., 2013; Sobrinho *et al*., 2013). In my allelopathic studies, aqueous and organic solvents rhizosphere soil, residues and root residues of *C. procera* in various experiments, invariably reduced the germination, plumule growth, radicle growth, dry weight, carbohydrate content, chlorophyll content, protein content and caused alteration of chromosome morphology of their respective recipient species [weeds (*Cassia tora* L., *Cassia sophera* L., *Chenopodium album* L., *Cannabis sativa* L.) and crops (*Pisum sativum* L., *Triticum aestivum* L., *Brassica oleraceae* var. *botrytis*, *Spinacia oleracea* L. and *Allium cepa* L.)]. The study suggests that *C. procera* is allelopathic plant, which is capable of suppressing the germination and growth of various test species. The allelopathic nature of *C. procera* reported by several authors is presented in Table 1. Besides, various allelochemicals such as calotropin, catotoxin, calcilin and gigantin have been identified (Daubenmire, 1974; Kuriachen and Dave, 1989). The allelopathic nature of *C. procera* lead to the discovery of new products (allelochemicals) with their evaluation as an alternate strategy for biological control of other plant and organisms (Hirai, 2003; Bhowmik and Inderjit, 2003; Belz, 2007; Macias *et al*., 2007; Norton *et al*., 2008). The allelopathic or invasive nature of *C. procera* is due to following features

- Adaptability of plant to a variety of soils and different environmental conditions.
- Non-consumption by grazing animals makes it to flourish rapidly (Oudhia *et al*., 1997).
- Its abundance in poor soils occurs when competition from native grasses has been removed by overgrazing (Smith, 2002).
- It grows in certain areas where nothing else grows and it becomes the only survivor (Sharma and Tripathi, 2009).
- It reproduces mainly by seed and often regrows from the root system when conditions become favorable for local increase in the size and density of populations.

- It forms pioneer vegetation in desert soil due to its drought tolerant ability (Smith, 2002).
- Xerophytic adaptations due to the presence of latex, extensively branched root system and thick leaves with waxy coverage also add to its invasive nature.
- The ability to compete with native vegetation, transform the appearance of the landscape, non-consumption by grazers and mustering declares it as a high priority weed (Crothers and Newbound, 1998; Csurhes, 2009).

Table 1: Allelopathic potential of *C. procera* reported by several authors.

Parts used	Parameters impacted	Target plants	References
Leaf aqueous extract	Final germination percentage decreased at higher concentrations, along with radicle length and plumule length	*Hordeum vulgare* L., *Triticum aestivum* L., *Cucumis sativus* L. and *Trigonella foenum-graecum* L.	Al-Zahrani and Al-Robai, 2007
Leaf and flower aqueous extract	Delayed germination, germination percentage, shoot length and increased proline content, particularly at higher concentration	*Triticum aestivum* L., *Raphanus sativus* L. and *Brassica napus* L.	Abdel-Farid *et al*., 2013
Aqueous shoot leachates	Chlorophyll, nitrogen percentage, protein percentage and mortality percentage were affected synergistically by 3rd and 9th day leachates of respective parts	*Parthenium hysterophorus* L.	Knox *et al*., 2010
Leaf water extract	Germination percentage, seed germination index, root length, shoot length seedling biomass and seedling vigor index were significantly reduced where as mean germination time and time to 50% germination were significantly increased	*Triticum aestivum* L.	Yasin *et al*., 2012
Leaf and stem aqueous extract	Germination, seedling growth, fresh and dry mass of the seedlings was inhibited significantly	*Pennisetum americanum* (L.) Leeke and *Setaria italic* (L.) Beauv.	Samreen *et al*., 2009

Leaf extract	Germination and seedling growth (root length, shoot length, fresh and dry weight of root and shoot) was enhanced	*Zea mays* L.	Naz and Bano, 2013
Leaf extract	Germination percentage, plumule and radicle length decreased significantly	*Cucumis sativus* L., *Lycopersicon esculentum* L. and *Solanum melongena* L.	Ghasemi *et al*., 2012
Aqueous leaf leachates	Germination, growth, coefficient of velocity (COV), plumule length, number of leaves, fresh weights of roots, shoots and dry weights of roots were significantly inhibited	*Glycine max* (L.) Merrill.	Ayeni and Akinyede, 2014
Aqueous leaf extract	Germination percentage, root length and shoot length of weed species decreased progressively when treated with increasing extract concentration (0.5,1,2 and 4%)	*Ageratum conyzoides* L., *Cannabis sativa* L. and *Trifolium repens* L.	Gulzar *et al*., 2014b

1.4. BACKGROUND ON CROPS AND WEEDS TO BE BIOASSAYED

1.4.1. Vegetable and cereals used for this study

In developing countries, vitamin deficiency and low micronutrients occur due to insufficient intake of required vegetables and consumption of predominantly starchy staples with or without animal products and a few vegetables and fruits in diets (Flyman and Afolayan, 2006). In addition, the cereals are good source of proteins and carbohydrates. As a means of eradication of poverty, hunger and malnutrition, there is a need for increased production of vegetables and cereals in many of the affected nations through government policies (Reardon *et al*., 2003). In Aligarh, vegetables such as tomato, spinach, cabbage, mustard, carrot, wheat and pea are common in many home gardens and cultivated fields. Hence the selection of cabbage (*B. oleracea*), spinach (*S. oleracea*), wheat (*T. aestivum*), pea (*P. sativum*) and onion (*A. cepa*) used in this study. The vegetables and cereals are short season and widely cultivated by the local people in cultivated fields where *C. procera* usually predominates.

1.4.2. WEEDS USED IN BIOASSAY

1.4.2.1. *Chenopodium album* (L.)

Chenopodium album L. family Chenopodiaceae, commonly known as lambsquarters or white goosefoot is one of the ten 'worst weeds', a native of Europe that has become established in different parts of the world and compete with the natural vegetation and crops (Holm *et al.,* 1997). Its ability to colonize the available niche in the invaded areas, its fast growth, huge monospecific strand formation (sometimes impenetrable) at the cost of the other natural vegetation, thereby affecting the native biodiversity describes its allelopathic nature (Batish *et al*., 2006a). In Aligarh, the weed grows profusely and invades winter-season crops like wheat, barley, rapeseed, vegetables, mustard, onion and severely affects their growth and yield (Shazia and Siddiqui, 2012). Under controlled laboratory and field conditions, several authors have reported its allelopathic impact (Bhowmik and Doll, 1979; Porwal and Gupta, 1986; Qasem and Hill, 1989; Bertin *et al*., 2003; Bais *et al*., 2004).

1.4.2.2. *Cassia tora* (L.)

Cassia tora L. family Caesalpiniaceae, commonly known as sickle senna grows throughout the tropical and subtropical regions of the world as an obnoxious, aggressive, annual, herbaceous weed. It shows common occurrence in warm, moist climates, especially those in the middle and southern parts of the India. Throughout the country, the plant has been referred to as the most economically destructive weeds (Oudhia, 1999). In Aligarh, it grows very aggressively, along roadsides, mostly invades cultivated fields, competing with crops for environmental resources and releasing toxic chemicals into the surrounding soil. The profuse growth of the plant and its competition with crops for environmental resources and released toxic chemicals into the immediate vicinity and its abundance along roadsides and cultivated fields is due to its allelopathic nature. Identification of a large number of compounds produced at a later stage of the plant also describes its allelopathic behavior (Sarkar *et al*., 2012). According to Inderjit (1996), water-soluble allelochemicals contribute to its allelopathic nature. The growth and metabolism of associated weeds also get affected by the aqueous extract of the whole plant, leaves and powdered leaves (Sarkar *et al*., 2012). The allelopathic impact of the weed has also been investigated by (Sarkar and Chakrabotry, 2011; Vitonde *et al*., 2014).

1.4.2.3. *Cannabis sativa* (L.)

Cannabis sativa L. family Cannabaceae, commonly known as bhang mainly suppressed crop plant and is declared as an effective weed for centuries (Willis, 2007). Its predominance is mainly attributed to its high competitiveness for water, food and light, rapid soil surface coverage makes hemp a strong weed suppressor, resulting in low herbicide requirements for hemp cultivation and therefore suppresses weed growth (Ranalli, 1999). It profusely grows in waste places and along roadsides and grows with rabi crops everywhere in Aligarh district. Its allelopathic activity against weeds has also been evaluated by crop rotation and related practices to the crops in rotation (Pudelko *et al*., 2014). Furthermore, in a crop rotation, this weed suppression effect can even improve the weed situation for the following crop (Werf *et al*., 1996; Struik *et al*., 2000; Robson *et al*., 2002). However, the aggressiveness and allelopathic effects on the neighboring plants is attributed due to its high relative dominance (Stupnicka-Rodzynkiewicz, 1970; Srivastava and Das, 1974; Bardi, 2002).

1.4.2.4. *Cassia sophera* (L.)

Cassia sophera L. family Caesalpiniaceae, commonly known as sickle senna extending from Africa to India and South East Asia believed to be native of South America. It is a common weed in uncultivated lands, along roadsides and on waste ground and describes its allelopathic nature (Mulay and Sharma, 2012). Its invasive nature is due to its fast growth rate, high reproductive and vegetative potential, adaptability to changing environmental conditions, wide ecological amplitude and allelopathy (Gulzar *et al*., 2014a). Allelochemicals (triterpenes) identified from *Cassia* are known for their allelopathic responses and great ecological significance with respect to invasion (Ghayal *et al*., 2007).

1.5. AIMS AND OBJECTIVES

The main aim of the study was to determine whether *C. procera* possess allelopathic potential, through the release of allelochemicals from different parts of the plant. The hypothesis is thus that *C. procera* produces compounds with allelopathic potential that affect the growth of surrounding plants, thereby gaining a competitive advantage.

Specific objectives were the following:

- To study the effect of rhizosphere soil of *C. procera* on the growth and establishment of recipient crops and weeds.

- To study the phytotoxic effect of aqueous extract of different parts (leaf, stem and root) of *C. procera* on the growth and establishment of recipient weeds and crops.
- To study the allelopathic effect of residue amended soil (RS), residue extract amended soil (RES) and residue extract (RE) on the growth and establishment of weeds and crops.
- Allelotoxicity of root residue amended soil (RRS), root residue extract amended soil (RRES) and root residue extract (RRE) in relation to growth and establishment of donor species have been studied.
- Extraction of allelochemicals from the leaves of *C. procera* and their impact on physiological parameters of selected crop and weed plants.
- Bio-efficacy of extracting allelochemicals on germination parameters of selected weed and crop plants.
- HPLC detection of allelochemicals from different parts of *C. procera* that contribute to its allelopathic nature.
- Foliar micromorphology of *C. procera* and their relation to allelopathy.
- Allelotoxic impact of *Calotropis* leaf aqueous extract on cytomorphology of bioassay plant *A. cepa*.

Chapter-2

Review of Literature

REVIEW OF LITERATURE

2.1. ALLELOPATHY AND ITS HISTORY

Although the allelopathy was first documented in approximately 300 B.C. by Greek and Roman writers, but the credit for providing the term allelopathy only goes to Hans Molisch in 1937 to describe the plant-plant interaction. Allelopathy is derived from the Greek words Allelon "of each other" and pathos "to suffer" (Rizvi *et al*., 1992). Allelobiogenesis or allelopathy defined as the combination of both biotic and abiotic stresses induced by donor plants on recipient plants. In the current literature, the term allelopathy is a chemical-mediated negative interference between plants or microbes through its direct or indirect effect (Rice, 1984; Willis, 2004; De Albuquerque *et al*., 2011; Yang *et al*., 2011).

The phenomenon of allelopathy has existed for thousands of years, for over 2000 years. However, recognition and understanding of allelopathy have occurred only by intensive scientific records over the past few decades (Weston, 2005). Theophrastus, "the father of Botany", wrote in his botanical works in 300 B.C. about the chickpea allelopathy and describes the weed and crop allelopathy according to earliest recorded observations (Khalid *et al*., 2002).

The history of allelopathy could be divided into 3 phases of its development (Singh *et al*., 2001).

(I) DeCandolle Phase

The period of late 18^{th} and early 19^{th} century, especially between 1785 and 1845,

(II) Pre-Molisch Phase

The period in the beginning of the 20^{th} century (from 1900-1920) known by the work of Pickering and Scheiner and

(III) Post-Molisch Phase

1937 onwards, which actually could progress since 1960 (Willis, 1997).

In communities, different plant species may interact in a positive, neutral or negative manner. Positive interaction includes obligatory or non-obligatory mutualism. Rarely, the organisms in a community remain neutral, especially when canopies and the roots of the plants occupy different niches. Negative interaction between the organisms are, however, more common. The adverse impact of a

neighboring plant in an association is termed interference (Muller, 1969). Putnam and Tang (1986) have categorized interference as:

(i) Allelospoly

More commonly called competition, which includes depletion of one or more resources acquired for the growth of organisms in an association.

(ii) Allelo-Mediation

Selective harboring of herbivore that might feed on one species, thus lending advantage to another (Szezepanski, 1977).

(iii) Allelopathy

Allelopathy, the chemical mechanism of plant interference, characterized by a reduction in plant performance in the association.

2.2. ALLELOPATHY IN NATURAL AND AGRICULTURAL ECOSYSTEMS

From the ecological perspective, allelopathy may play a role in biological invasion, plants use allelochemicals as weapons against native plants, which are sensitive to these chemical compounds to which coexisting species are, on the contrary, adapted (Callaway and Ridenour, 2004). Indeed, allelopathy has been reported as a result of long-term co-evolution within established plant communities and it may have a maximum retardatory effect on newly introduced species (Mallik and Pellissier, 2000). Ecosystem processes and structures can be affected by plant secondary metabolites and allelopathy can be used as a natural strategy protecting plants against environmental ‘‘enemies’’ and competing plants (Prince and Pohnert, 2010)

Allelopathy has to be taken into consideration also in agricultural ecosystems, when considering the cultivation of different plants together or in succession (Scognamiglio *et al*., 2013). However, the potential of allelochemicals to act as a substitute for natural herbicides have received much attention. In fact, due to their natural origin, many researchers have suggested that most allelopathic compounds are more biodegradable and less polluting than traditional herbicides (having shorter life time), offering also a clue against the emergence of weeds resistant to older synthetic molecules (Reigosa *et al*., 2006). The consideration that allelopathy can be a viable component of crop/weed interference, prompted the idea of exploiting this phenomenon to manage weeds (Belz, 2007; Farooq *et al*., 2011). Allelochemical influence the success of practices like crop rotation and intercropping, while weed

suppression ability of allelopathic species is based on the use of cover or smother crops. Allelochemicals released by living plants (Weston and Duke, 2003) or allelopathic crop water extracts (Farooq *et al*., 2011) have been reported as strategies in weed management. Commercially available mixtures of organic natural products include the mixture of vinegar, lemon extract and clove oil. Maize gluten meal is also used as herbicide and fertilizer, its herbicidal effect is due to phytotoxic diterpenes and to a pentapeptide (Liu and Christian, 1996). Moreover, it should not be ignored that, even if they are ‘‘natural’’, these products can anyway be toxic, fumonisin is toxic to mammalian cells (Duke *et al*., 2000b), while sorgoleone is reported to cause dermatitis (Inderjit and Bhowmik, 2002)

2.3. METHODOLOGICAL APPROACHES USED IN ALLELOPATHIC STUDIES

Allelopathy is a very complex phenomenon with several nuances and a web of interconnections with a number of ecological and physiological processes (Scognamiglio *et al*., 2013). It follows that its elucidation cannot be easily achieved. For allelochemical identification phytotoxicity determination can be the first step, but it is not enough to state that an allelopathic interaction has been determined. Various approaches in allelopathy studies have been reported in the literature and each one is able to meet specific requirements regarding few aspects. The optimum method setup is of paramount importance when defining allelopathic interactions that can be elucidated only thanks to well-designed and interlinked bioassays and field experiments (Inderjit and Callaway, 2003).

A detailed survey of all of the methods goes beyond the goal of this review, but, as the distinction between phytotoxic and allelopathic activity depends to a great extent on the used approach, a brief overview is needed. Methods used for allelopathy (laboratory and in field bioassays) have been reviewed by Wu *et al*. (2001), Inderjit and Callaway (2003), Inderjit and Nilsen (2003), while the related problems are widely discussed by Inderjit and Dakshini (1995), Inderjit and Weston (2000).

Traditional approaches to discover and assess allelochemicals are based on bioassay-guided fractionation. A number of separation methods together with spectroscopic techniques gave very good results (Macias *et al.,* 1999). Many studies deal with the extraction of plant material with a solvent, a mixture of solvents or a series of them. For allelopathy studies, the most used solvent is water, as it is

supposed to simulate natural conditions. This is true, but extraction conditions are already far from natural systems, where allelochemicals are released into the environment in several ways, depending on their physico-chemical properties and on the plant organ involved. Plant extracts are complex mixtures, being made up by a number of secondary metabolites and synergistic or/and additive effects are often supposed or demonstrated to exist (Reigosa *et al*., 1999b). In many reports, putative allelochemicals are identified directly in mixtures or in partially purified fractions by HPLC (Chon and Kim, 2002; Thi *et al*., 2008) or GC–MS (Bousquet-Melou *et al*., 2005). When the goal is the isolation of active compounds, extracts undergo simple bioassay and they are further partitioned if active. The fractions are tested and the active ones purified. In some studies pure active compounds are identified, isolated and characterized and tested again for their phytotoxic potential (Chon and Kim, 2002; Kato-Noguchi *et al*., 2012) but this is not always feasible. This bioactivity-guided fractionation of extracts led to the isolation of several phytotoxic compounds. The determination of phytotoxic activity relies on specifically designed tests (Table 2).

Table 2: Methods used in allelopathic studies (Scognamiglio *et al*., 2013).

Method	Method Description	Matrix	References
Petri dishes assays	Petri dishes bottom lined with filter paper or solid agar medium with receiving plant Seeds	Extracts	Chon and Kim 2002, Shirahishi *et al.*, 2002; Hao *et al.*, 2007; El Marsni *et al*., 2011, Valera-Burgos *et al*., 2012
	Measurements (germination velocity, rate and percentage, shoot and root length) carried out after some days of incubation		
		Partially purified fractions	El Marsni *et al*., 2011, KatoNoguchi *et al.*, 2012
		Radicle exudates	Shirahishi *et al.*, 2002; Chon and Kim, 2005, Hao *et*

			al., 2007
		Essential oils	Verdeguer *et al*., 2009, Silva *et al.*, 2012
		Pure compounds	Reigosa *et al.*, 1999b; Macias *et al*., 2000; Chon and Kim, 2002, Reigosa and Pazos-Malvido, 2007, Scognamiglio *et al*., 2012c
	Soil tested directly	Soil allelopathy	Herranz *et al.*, 2006
	Co-sown donor and receiving	Plant direct plant-plant interaction	Emeterio *et al.*, 2004
Wheat coleoptile assay	Quantification of the wheat apical zone elongation in a liquid medium in the presence of allelochemicals or fractions	Extracts, partially purified fractions or pure compounds	El Marsni *et al.*, 2011
Hydroponic and in pot test	Like Petri dishes assays but with older plants	Extracts, partially purified fractions or pure compounds	Nimbal *et al.*, 1996; Josie and Gillepsie, 1998; Kim *et al.*, 2005; El Marsni *et al*., 2011; Hussain and Reigosa, 2011
	Morphological changes and physiological responses measured		
	Isolation of root exudates	Root exudates	Esmaeili *et al.*, 2012a
	Observe the effects on the test plant while monitoring exudate chemical composition	Root exudates	Belz and Hurle, 2004
CRETS (Continuous root exudates trapping system)	Seedlings grown in hydroponic nutrient solution on line with a column containing XAD-4 resin, which traps	Root exudates	Tang and Young, 1982; Hao *et al.*, 2007

	allelochemicals exudate from roots		
Plant box method	Donor plant root put on one corner of a plant box filled with agar and test seeds placed on agar gel substrate, in order to study the effects of root exudates on seed germination and seedling growth	Root exudates	Shirahishi *et al.*, 2002; Fujii *et al*., 2003
Bioassay in rotation	Donor plant seeds sown on wool rock block and glass perlite with hoagland solution	Root exudates	Macias *et al.*, 2004
Box growth method paired with root image analysis system	Use of the digital camera technology to measure root length, spread and surface (dynamics) of plants growing in a glass container	Co-growth of donor and receiving plants	Mardani and Yousefi, 2012
Dish pack method	Plant material put into one hole of a 6-well multi-dish, other holes with test seeds. Seed germination and growth measured. Volatiles analysed by GC–MS	Volatile allelochemicals	Fujii *et al.*, 2005a
Plant sandwich method	Inclusion of plant material into two layers of agar medium (or quartz) used to grow the test plant	Leachates from litter or decomposing plant material	Shirahishi *et al*., 2002; Kato-Noguchi, 2003; Fujii *et al.*, 2003; Fujii *et al.*, 2004; Morikawa *et al*., 2012
Agar solid tissue culture method	Donor plant powder mixed with agar	Leachates from litter or decomposing plant material	Zuo *et al.*, 2012
ECAM (Equal compartment agar medium)	Donor seeds sown on agar surface in one half of a glass beaker prefilled agar	Co-growth of donor and receiving plants	Wu *et al.*, 2000

Greenhouse pot bioassays (or in field)	Plant material spread over soil surface	Leachates from litter or decomposing plant	Thi *et al.*, 2008
	Amendment of plant residues	Leachates from litter or decomposing plant material	Chon and Kim, 2005; Batish *et al.*, 2006a, 2007b, Matloob *et al.*, 2010
	Amendment of plant residue extracts	Plant residue extracts	Singh *et al.*, 2003c
	Amendment of pure compounds	Pure compounds	Bertin *et al.*, 2009
	Co-growth of donor and receiving plant	Interaction donor/ receiving plant	He *et al.*, 2009; Viard-Cre tat *et al.*, 2009; Labbafi *et al.*, 2010
	Field studies	Various	Akemo *et al.*, 2000; Pheng *et al.*, 2009; Klionsky *et al.*, 2011, Tesio *et al.*, 2011
Allelochemical soil static concentration determination	Solvent extractions of chemical compounds from the soil matrix paired with HPLC analysis	Allelochemicals in the soil	Scognamiglio *et al.*, 2012b
Solid-phase root zone extraction (SPRE) method	Use of sorbent material probes placed in the soil	Allelochemicals in the soil	Weidenhamer *et al.*, 2009
In situ silicone tube microextraction method	Use of sorbent material probes placed in the soil	Fate of allelochemicals in the soil	Mohney *et al.*, 2009
Rhizosphere soil method	Modified sandwich method, the soil surrounding roots used for inclusion in agar	Allelochemicals in the soil	Fujii *et al.*, 2005b
Allelochemical solid-phase microextraction	SPME (Solid phase microextraction) method SPME fibre inserted into the stem of the test plant and the adsorbed compounds can be	Allelochemical uptake	Loi *et al.*, 2008

	analysed by GC or HPLC		
Radiolabeled allelochemicals	Observing the compound fate within the plant	Allelochemical uptake	Chiapusio *et al.*, 2004
Flow cytometry	Detection of effects on cell cycle	Allelochemical mode of action	Zhang *et al*., 2010b
Confocal microscopy	Detection of effects on target cells	Allelochemical mode of action	Chaimovitsh *et al.*, 2010
Physiology measurements	Measurement of several parameters	Allelochemical mode of action	Hussain and Reigosa, 2011
Use of silenced plants	Plant ability to synthesize and release specific modified	Allelochemical production	Inderjit *et al*., 2009
Metabolomics approach	Determination of plant metabolome	Allelochemical production	Scognamiglio, 2011
	Plant extract tested on a test species	Allelochemical effect on test species and putative mode of action	
	Test species metabolomic analysis		

2.4. REPORTS ON ALLELOPATHIC WEEDS FROM 2006

Since, there have been several reports on the allelopathic studies of weeds. It is primarily due to the availability of modern instrumentation, suitable techniques for the identification, extraction and characterization of the allelochemicals involved. In addition, under natural and managed conditions, there has been an increase in the number of studies demonstrating the phenomenon. Here is a long list of available reports on the allelopathic potential of weeds in the agroecosystems from 2006 tabulated below in Table. 3.

Table 3: List of weeds exhibiting allelopathic effects on other plants (Reports from 2006).

Source	Target plant	Part used and its effect	Reference
Artemisia harba- alba Asso.	*Anabasis setifera* Moq.	Aqueous extract of mature and immature fruit inhibited the germination percentage and seedling growth	Modallal and Charchafchi, 2006
Acacia	*Carrichtera annua* (L.)	Aqueous extract of	Dana and

retinodes Schltdl., *Euphorbia serpens* L. and *Nicotiana glauca* Graham	Dc., *Conyza albida* Willd. ex Spreng, *Lactuca sativa* L. and *Lycopersicon esculentum* L.	whole plant inhibited the root length and shoot length	Domingo, 2006
Achillea biebersteinii Afan.	*Capsicum annuum* L.	Germination percentage, germination rate, radicle length, shoot length, chlorophyll a, chlorophyll b, total chlorophyll,carotenoids and protein were significantly reduced in response to allelochemical stress of leachates	Abu-Romman, 2011
Achillea santolina L.	*Vicia faba* L. and *Hordeum vulgare* L.	Aqueous shoot extracts decreased chlorophyll a and b levels, increased carotenoid content, increased levels of CAT, GPX, SOD and GR activity while residues and aqueous extract of residue inhibited seedling length	Darier and Tammam, 2012
Ageratium conyzoides L., *Cynodon dactylon* (L.) Pers, *Parthenium hysterophorus* L. and *Solanum nigrum* L.	*Glycin max* (L.) Merrill.	Extract of whole plant inhibited the protein content, protein profile, seed germination and seedling length	Verma and Rao, 2006
Ageratum conyzoides L.	*Raphanus sativus* L.	Phytotoxicity of below-ground residues changes during decomposition and was reduced upon the addition of soil to the residues	Kaur *et al*., 2012
Ageratum conyzoides L.	*Cicer arietinum* L.	Root length, plant height, biomass, nodule number, weight and leghemoglobin content were lower in the soils amended with below or above-ground weed residues.	Batish *et al.*, 2006b

Ageratum conyzoides L.	*Oryza sativa* L.	Seedling length and dry weight were significantly reduced in rhizosphere soil, in leaf debris and debris extract amended soil	Batish *et al*., 2009a
Ageratum conyzoides L.	*Oryza sativa* L.	Root exudates and residues suppress the growth by releasing phenolic allelochemicals into the rhizosphere soil	Batish *et al*., 2009b
A*geratum conyzoides* L.	*Vigna radiata* (L.) R. Wilczek and *Vigna mungo* (L.) Hepper.	Various concentrations of whole plant extracts gradually reduced the germination percentage, seedling length, dry weight, photosynthetic pigments, protein and amino acid contents	Jayaraman and Ramalingam, 2014
Ageratum conyzoides L. and *Cleome viscosa* L.	*Sesamum indicum* L.	Inhibitory effect on germination percentage, root growth, shoot growth, fresh and dry weight	Natarajan *et al*., 2014
Amaranthus hybridus L.	*Phaseolus vulgaris* L.	Aqueous extracts affected relative water content (RWC), both vegetative growth and grain yield	Amini and Ghanepour, 2013
Amaranthus retroflexus L., *Chenopodium album* L., *Erigeron canadensis* (L.) Cronquist and *Solanum nigrum* L.	*Glycine max* (L.) Merrill., *Pisum sativum* L. and *Vicia sativa* L.	Extract from fresh and dry biomass had inhibitory effect on seed germination	Marinov-Serafimov, 2010
Ambrosia artemisiifolia L	*Medicago sativa* L., *Hordeum vulgare* L., *Zea mays* L., *Lactuca sativa* L., *Lycopersicon esculentum* L., *Triticum aestivum* L., *Echinochloa crus-galli* (L.) P. Beauv, *Solanum nigrum* L., *Portulaca oleracea* L., and *Digitaria sanguinalis* (L.) Scop.	Effect of residue and root exudates reduced germination, inhibited shoot and root growth. However, the response of test species varies	Vidotto *et al*., 2013

Ambrosia trifida L.	*Triticum aestivum* L.	Growth gets significantly inhibited in infested or residue amended soils	Kong *et al*., 2007
Apilia africana (Pers.) C. D. Adams, *Emilia sonchifolia* (L.) DC. ex Wight, *Crotalaria retusa* L., *Chromolaena odorata* L., *Panicum maximum* L. and *Cyperus esculentus* L.	*Zea mays* L., *Citrullus lanatus* Thunb, *Abelmoschus esculentus* L. Moench, *Vigna unguiculata* L. Walp, *Glycine m*ax L. Merr. and *Arachis hypogaea* L.	Both the water extracts and the decomposing mulches of tops of all the test weeds significantly inhibited the germination to varying degrees	Usuah *et al*., 2013
Aristolochia esperanzae Kuntze	*Sesamum indicum* L.	Extracts caused marked changes in germination, seedling growth and 50% reduction in the size of root xylem cells and marked changes in the primary root and in the number of secondary roots	Gatti *et al*., 2010
Artemisia annua L.	*Helianthus annuus* L., *Lactuca sativa* L., *Zea mays* L., *Amaranthus retroflexus* L., *Echinochloa crus-galli* (L.) P. Beauv and *Lolium perenne* L.	Leaf extract reduced the germination of both (weed and crop) test plants	Koloren, 2006
Avena fatua L. and *Secale cereale* L.	*Triticum aestivum* L.	Plant debris and mulch management has a significant effect on germination parameters	Amoghein *et al*., 2013
Bothriochloa laguroides var. *laguroides* **(**DC.) Herter	*Lactuca sativa* L., *Zea mays* L., *Paspalum guenoarum* Arechav. and *Eragrostis curvula* (Schrad.) Nees	Stem and leaf extracts caused inhibition of root and shoot elongation in all four species tested. Aqueous extracts were generally less inhibitory to seed germination	Scrivantia, 2010
Cannabis sativa L.	*Triticum aestivum* ssp. *vulgare* L., *Secale cereale* L., *Lupinus luteus* L. and *Brassica napus* ssp. *oleifera* L.	Germination, length, weight and number of roots and leaves decreased by aqueous extract	Pudelko *et al*., 2014

Cassia sophera (L.) Roxb	*Chenopodium album* L., *Melilotus alba* Medik and *Nicotiana plumbaginifolia* Viv.	Aqueous extract at different concentration reduced the germination percentage, seedling growth, dry biomass, leaf area, relative water content, total protein and chlorophyll content	Gulzar *et al*., 2014a
Cassia tora L.	*Brassica juncea* (L.) Coss	Aqueous extract from root, stem and leaf reduced seed germination, root length, shoot length, chlorophyll content, fresh weight (FW), dry weight (DW) and relative water content (RWC)	Sarkar *et al*., 2012
Chenopodium murale L.	*Triticum aestivum* L.	Rhizosphere soil and root residues exhibit inhibitory effect on seedling length and seedling dry weight. Only a partial amelioration in growth inhibition occurred upon charcoal supplementation or nitrogen fertilization in these amended soils	Batish *et al*., 2007a
Chenopodium album L.	*Triticum aestivum* L.	Concentrated leaf extracts had detrimental effects on plant height, number of tillers and spike length	Majeed *et al*., 2012
Chenopodium album L., *Amaranthus retroflexus* L. and *Cynodon dactylon* L.	*Carthamus tinctorius* L.	Weed extracts significantly decreased plant height and root dry weight	Rezaie and Yarnia, 2009
Chenopodium murale L.	*Cicer arietinum* L. and *Pisum sativum* L.	A significant reduction in root and shoot length as well as dry matter, root oxidizability, occurred when both the legumes were grown in the soil amended residue. A significant decline in nodule	Batish *et al*., 2007b

		number, weight and leghaemoglobin content was recorded	
Chenopodium murale L.	*Oryza sativa* L.	Combination of leaf extract and NaCl drastically reduced the shoot and root growth more than the separate effects of these stress treatment	Alam and Shaikh, 2007
Chenopodium murale L. and *Malva parviflora* L.	*Hordeum vulgare* L.	A clear effect of extract was recorded on the growth parameters, plant height, number of leaves, number of tillers, root fresh and dry weight.	Al-johani *et al*., 2012
Chenopodium album L.	*Cassia occidentalis* L. and *Phaseolus aureus* L.	Phenolics released from above and belowground parts, rhizosphere soil and debris amended soil probably involved in the growth retardatory effect	Batish *et al.*, 2006a
Chromolaena odorata (L.) King and Robinson and *Mikania micrantha* Kunth	*Ageratum conyzoides* L., *Eleusine indica* (L.) Gaertn. and *Cyperus iria* L.	Aqueous leaf extract and leaf debris significantly reduced all seedling growth parameters	Sahid and Yusoff, 2014
Chrysanthemoids monilifera ssp. *monilifera*	*Lactuca sativa* L., *Isotoma axillaris* Lindl. and *Acacia mearnsi* De Wild.	Aqueous extracts of organs showed ranked inhibition similar to phenolic content on germination indices, biometric, physiological and biochemical parameters	Al Harun *et al*., 2014
Chrysopogon serrulatus Trin.	*Leptochloa chinensis* (L.) Nees	Allelochemicals showed different degrees of inhibitory effects on germination, shoot and root growth	Chuah *et al*., 2014
Cleome arabica L. and *Capparis spinosa* L.	*Lactuca sativa* L.	Aqueous and methanol extracts affected cytological, physiological and biochemical processes	Ladhari *et al*., 2014

Coronopus didymus L.	*Triticum aestivum* L.	Variable phytotoxicity was exhibited by different extract sources and leaf extract caused the greatest inhibition	Khaliq *et al*., 2013b
Cronopus didymus L. Sm.	*Oryza sativa* L.	Emergence, seedling growth and chlorophyll content decreased with increasing concentration of residue amendation in soil	Khaliq *et al*., 2013a
Croton bonplandianium Baill.	*Parthenium hysterophorus* L.	Leaf residue in soil inhibited the seed germination and seedling growth	Thaper and Singh, 2006
Croton bonplandianum Baill.	*Triticum aestivum* L., *Brassica rapa* L., *Brassica oleracea* var. *botrytis* L. , *Spinacea oleracea* L., *Melilotus alba* Medik., *Vicia sativa* L. and *Medicago hispida* Gaertn.	Root length, shoot length and dry weight of seedlings decreased significantly when plants were grown in rhizosphere soil	Sisodia and Siddiqui, 2009
Cymbopogon nardus (L.) Rendle	*Medicago sativa* L., *Lepidum sativum* L., *Lactuca sativa* L., *Echinochloa crus-galli* (L) P. Beauv, *Lolium moltiflorum* Lam. and *Echinochloa colonum* L.	Inhibitory activity of leaf and root extracts was more effective than stalk extract, besides the response of allelochemical stress also varies among species	Suwitchayanon and Kato Noguchi, 2014
Cynanchum acutum L.	*Triticum aestivum* L.	Increasing the concentration rate of watery distillate, germination percentage, radicle and shoot length reduced	Golzardi *et al*., 2014
Cyperus iria L.	Five varieties of rice MR211, MRQ74, MR220, MR84 and MR232	Leaf and stem extracts showed comparatively higher inhibitory effects on seedling growth, reduced chlorophyll content as compared to root extract. The sensitivity also varies among varieties	Ismail and Siddiqui, 2011
Cynoglossium officinale L.	*Agropyron cristatum* (L.) Gaertn., *Elymus wawawaiensis* J. Carlson & Barkworth var. *Secar*,	Leaf extracts and residues inhibit seed germination, seedling emergence and UV-B	Furness *et al*., 2008

	Festuca idahoensis Elmer var. *Joseph, Koeleria macrantha* (Ledeb.) J.A. Schultes	may enhance their allelopathic influence on some forage grasses	
Cynodon dactylon L	*Zea mays* L.	Seed germination and plant growth delayed at the higher concentrations	Bibak and Jalali, 2016
Cynodon dactylon (L.) Pers. and *Alternanthera sessilis* (L.) R.Br.	*Sorghum vulgare* Pers.	Allelopathic potential was investigated in relation of seed germination, root-shoot length, biomass and protein conten upon exposure to leaf aqueous extract	Mali and Kanade, 2014
Descurainia sophia (L.) Webb ex Prantl	Wheat cultivars (Huaimai 20, Fengyuan 1, Zhongmai 1, Duokang 1, Baomai 1, Zhou 18, Zhou 16 and Wen 10)	Seed germination was inhibited. Seedling shoot and root growth (in terms of both length and dry weight) were also significantly inhibited in response to the volatile aqueous solution compared to the control	Li *et al*., 2011b
Echinochloa crus-galli (L.) P. Beauv	Rice genotypes *(*IR60, Sefidrood, Khazar, Nemat, Neda, Fajr, Tarom, Shiroodi and Hybrid)	Leaf extract had the highest inhibitory activity on root lengths depending upon individual ability of rice genotypes	Esmaeili *et al*., 2012b
Echinochloa colona L., *Cleome viscosa* L. and *Ammania baccifera* L.	*Vigna radiata* (L.) Wilczek	Degree of reduction percentage of all the growth parameters (germination, seedling growth, dry weight) was concentration dependent	Manikandan and Prabhakaran, 2014
Eclipta alba (L.) Hassk.	*Arachis hypogaea* L. and *Vigna radiata* L.	Rhizosphere soil exerts an allelopathic interference by releasing water soluble phenolic acids (vanillic acid, benzoic acid, ferulic acid and *p*-coumaric acid) in soil	Gulzar and Siddiqui, 2015

Eclipta alba (L.) Hassk.	*Amaranthus spinosus* L., *Cassia tora* L. and *Cassia sophera* L.	Aqueous leachate and organic fractions reduced the level of biochemical activities (Carbohydrate, chlorophyll and protein content)	Gulzar and Siddiqui, 2014b
Eclipta alba (L.) Hassk.	*Cassia tora* L., *Cassia sophera* L., *Phaseolus aureus* L. and *Oryza sativa* L.	Root length, shoot length and dry biomass of test species decreased progressively with increasing concentration (0.5,1,2 and 4%) of aqueous extract	Gulzar and Siddiqui, 2014a
Erythroxylum monogynum Roxb.	*Solanum lycopersicum* Mill. var. PKM-1	Seed germination, plumule length, radicle length, fresh weight and dry weight were notably decreased by leaf and stem extracts	Alagesabop athi, 2014
Eupatorium adenophorum Spreng.	*Amaranthus retroflexus* L. and *Chenopodium glaucum* L.	Inhibitory effects of leachates on membrane permeability, germination, growth and physiological characteristics of the seedlings occurred at highest concentration with prolonged treatment period	Jinhu *et al*., 2012
Eupatorium adenophorum Spreng., *Ageratum conyzoides* L. and *Lantana canara* L.	*Triticum aestivum* cv. HPW-42, *Oryza sativa* cv. Hasanshrasativa I Basmati and *Zea mays* cv. Girija, *Oryza sativa* L.	Incorporation of weed residue in soil had inhibitory effect on percent germination, shoot length and physiology	Katoch *et al*., 2012
Euphorbia guyoniana Boiss. & Reut.	*Bromus tectorum* L., *Melilotus indica* (L.) All. and *Triticum aestivum* L.	Germination efficiency, plumule and radicle length reduced upon exposure to aqueous extract	Nasrine *et al*., 2013
Euphorbia helioscopia L.	*Triticum aestivum* L., *Cicer arietinum* L. and *Lens culinaris* Medic.	Seedling emergence, seedling vigor index and total dry weight were significantly reduced upon exposure to rhizosphere soil and aqueous extract of various organs	Tanveer *et al*., 2010

Euphorbia himalayensis (Klotzsch) Boiss.	*Triticum aestivum* L., *Lactuca sativa* L. *Poa annua* L., *Festuca rubra* L. and *Trifolium pratense* L.	Root exudates from rhizosphere soil exhibited allelopathic activities	Liu *et al*., 2014
Festuca paniculata L.	*Fescuta paniculata* L., *Dactylis glomerata* L. and *Bromus erectus* Huds.	Leachates inhibited seedling growth was correlated with polyphenol concentration	Viard-Cretat *et al*., 2009
Fimbristylis miliacea (L.) Vahl	Rice varieties MR211, MRQ74, MR220 and MR84	Aqueous extract, weed debris and root exudates exhibit inhibitory effect on root growth, shoot growth, plant height and dry biomass	Ismail and Siddique, 2012
Heracleum mantegazzianm Somm. et Lev.	*Lolium perenne* L. and *Brassica napus* L.	Leachates inhibited seed germination and the level of inhibition was concentration dependent	Balezentiene and Renco, 2014
Hordeum spontaneum Koch.	*Triticum aestivum* L.	High residue levels amended in soil and exudates from tillers reduced mature plant height, fresh, dry weights and yield	Hamidi *et al*., 2008
Hyptis suaveolens (L.) Poit.	*Parthenium hysterophorus* L.	Seed germination and other biochemical components were decreased with increased concentration of leaf leachates and extracts, soaking period and exposure period	Kapoor, 2012
Inula crithmoides L.	*Raphanus sativus* L., *Lactuca sativa* L., *Peganum harmala* L. and thistle	Soil incorporation of residues significantly decreased root and shoot length. Irrigation soil with leaves and flower aqueous extracts was harmful	Omezzine *et al*., 2011
Jasminum officinale f. var. *grandiflorum* (Linn.) Kob.	*Echinochloa crus-galli* (L.) Beauv	Inhibitory effect of methanolic extract on germination, seedling growth, imbibition and α-amylase activities	Teerarak *et al*., 2012
Jasminum officinale f. var. *grandiflorum* (Linn.) Kob.	*Echinochloa crus-galli* (L.) Beauv., *Phaseolus lathyroides* L. and *Allium cepa* L.	Methanolic extract from dried leaves inhibited seed germination and stunted both root and	Teerarak *et al*., 2010

		shoot length. Likewise, the mitotic phase index was altered in onion incubated with crude extract	
Lantana camara L.	*Lens esculanta* Moench.	Phytotoxicity of rhizosphere soil were investigated under in vivo and in vitro conditions with effect on root, shoot elongation and seed germination percentage	Singh *et al*., 2012
Leonurus sibirics L.	*Lolium multiflorum* Lam., *Echinochloa crus-galli* L., *Lepidum sativum* L., *Lactuca sativa* L., *Phleum pretense* L., *Digitaria sanguinalis* L. scop., *Medicago sativa* L. and *Brassica napus* L.	Inhibitory activities of the extracts depended on the concentrations of the extract and test plant species	Islam and Kato-Noguchi, 2014a
Leonurus sibiricus L.	*Raphanus sativus* L., *Lactuca sativa* L. and *Lepidium sativum* L.	Methanol extract of leaves caused significant reduction only in the germination of *Lactuca sativa*, with no effects on the germinative processes of *Raphanus sativus* and *Lepidium sativum*	Rolim de Almeida *et al*., 2008
Lepidium sativum L.	*Amaranthus caudatus* L. and *Lactuca sativa* L.	Both species grew longer hypocotyls and shorter roots in presence of live cress seedlings. Seed exudate increased epidermal cell number, elongated cells of hypocotyl, with smaller hypocotyls circumference but greater epidermal cell number counted round the circumference	Iqbal and Fry, 2012
Lomandra longifolia Labill.	*Lactuca sativa* L.	Root exudates in soil reduced the growth	Asao *et al*., 2007
Medicago sativa L. and *Vicia cracca* L.	*Amaranthus retroflexus* L. *Lolium perenne* L., *Ipomea hederacea* [*Pharbitis hederacea* L.]	Leaf and root extract inhibited the germination and growth	Koloren, 2007

	and *Portulaca oleracea* L.		
Nicotiana plumbaginifolia Viv.	*Zea mays* L. cv. Uttam	Aqueous leachates reduced the germination rate (GR), stimulated the amylase activity and resulted in higher sugar content and GR, decreased the amount of chlorophyll a and b, carotenoids, protein and nitrate reductase activity (NRA), stimulated the activity of peroxidase, superoxide dismutase and catalase exhibited concentration dependent increase	Singh *et al*., 2009
Nigella sativa L.	*Lactuca sativa* L.	Aqueous extracts of seeds and aerial parts harvested at vegetative, flowering and fruiting significantly delayed germination, reduced its rate and affected seedling growth	Zribi *et al*., 2014
Ocimum tenuiflorum L.	*Lepidium sativum* L., *Lactuca sativa* L., *Medicago sativa* L., *Lolium multiflorum* Lam., *Echinochloa crus-galli* (L) P. Beauv and *Phleum pretense* L.	Plant extracts reduced significantly the total germination percent (GP), germination index (GI), germination energy (GE), speed of emergence (SE), seedling vigour index (SVI) and coefficient of the rate of germination (CRG) in addition to root and shoot growth	Islam and Kato-Noguchi, 2014b
Parthenium hysterophorus L.	Maize hybrid (DK 6142)	Aqueous extract and rhizosphere soil reduce the germination percentage, germination index, germination energy, seedling length, seedling biomass and seedling vigor index	Safdar *et al*., 2014
Parthenium hysterophorus L.	*Brassica rapa* L.	Aqueous extract of green leaf and flower reduced the seed germination, survival,	Prasad and Priyadarshani, 2006

		cotyledon area, leaf number, branch number, plant height, root weight, fertilization value and pollen viability	
Parthenium hysterophorus L. and *Chromolaena odorata* (L.) King and Robinson	*Zea mays* L.	Seed germination, radicle and plumule growth on account of allelopathic inhibition was observed at highest concentration of leaf extract	Devi and Dutta, 2012
Parthenium hysterophorus L.	*Oryza sativa* L., *Zea mays* L., *Triticum aestivum* L., *Aphanus sativus* L., *Brassica campestris* L., *Brassica oleracea* L., *Artemisia dubia* Wall ex. Besser and *Ageratina adenophora* (Spreng.) King and HE Robins	Extract had strong inhibitory effect to root and shoot elongation	Maharjan *et al*., 2007
Parthenium. hysterophorus L.	*Cassia occidentalis* L., *Cassia sophera* (L.) Roxb and *Cassia tora* L.	Aqueous extract of inflorescence, stem and leaf inhibited the seed germination and seedling growth	Rahman, 2006a,b
Peganum harmala L.	Dicot plants (lettuce and amaranth) and monocot plant (wheat and ryegrass)	Main alkaloid harmaline inhibit root length and shoot length especially dicots, inhibiting root elongation at a very low concentration where as harmine exhibited much weaker non-selective inhibitory effect	Shoa *et al*., 2013b
Pueraria montana Lour.	*Lactuca sativa* L., *Raphanaus sativus* L., *Bidens pilosa* L. and *Lolium perenne* L.	Both leaf, root extracts, litter and rhizosphere soil significantly inhibited germination indices (total germination, speed of germination and coefficient of the rate of germination)	Rashid *et al*., 2010a
Phalaris minor L., *Chenopodium murale* L.,	*Triticum aestivum* L.	Aqueous extracts caused inhibitory effects on seed germination, seedling length and	Ankita and Chabbi, 2012

Sonchus oleraceus L., *Cyanodon dactylon* L. and *Convolvulus arvensis* L.		seedling dry weight which increased progressively on increasing the concentration of weed plant part extracts	
Phalaris aquatica L.	*Chloris truncate* R. Br., *Trifolium subterraneum* L., *Medicago trunculata* Gaertn. and *Phalaris aquatica* L.	Aqueous extract neither inhibited germination nor impacted on radicle length, however exhibited autotoxicity by inhibiting radicle length	Adams *et al*., 2010
Pituranthos tortuosus (Coss.) Maire	*Linum usitatissimum* L.and *Brassica rapa* L.	Significant growth inhibition of target species was recorded in the presence of the aqueous and acetone extracts, more than 50% in certain cases	Krifa *et al*., 2011
Pluchea dioscoridis (L.) DC.	*Corchorus olitorius* L., *Lepidium sativum* L. and *Cynodon dactylon* (L.) Pers.	Soil obtained from the rhizosphere zone caused significant growth reductions	Fahmy *et al*., 2012
Poa annua L., *Imperata cylindrica* (L.) Beauv., *Cirsium arvense* (L.) Scop., *Datura alba* Nees and *Phragmites australis* (Cav.) Steud.	*Zea mays* L., *Avena fatua* L., *Convolvulus arvensis* L., *Ammi visnaga* L., *Rumex crispus* L. and *Asphodelus tenuifolius* Cav.	Germination, shoot weight and shoot length reduced. Differential response was recorded for different weeds extracts	Khan *et al*., 2011a
Polygonum hydropiper L., *Amaranthus spinosus* L., *Chenopodium album* L., *Cyperus rotundus* L. and *Imperata cylindrica* L.	Corn (cv. Barnal)	Germination, seedling growth and dry matter production affected by both the dried plant parts and aqueous extracts	Samad *et al*., 2008
Pueraria montana (Lour) Merr.	*Bidens pilosa* L. and *Lolium perenne* L.	Root length, shoot length, dry weight and chlorophyll concentration gets significantly affected	Rashid *et al*., 2010b

		when they were grown in litter leachate-amended soil	
Rhazya stricta Decne.	*Zea mays* L.	Aqueous extracts present inhibitory effects on germination and seminal root numbers, while leave extract significantly decreased the plumule and radicle growth	Khan *et al*., 2011b
Rheum emodi L., *Saussurea lappa* Clarke and *Potentilla fulgens* L.	*Amaranthus caudatus* L., *Phaseolus mungo* L., *Phaseolus vulgaris* L., *Eleusine coracana* (L.) Gaertner, *Triticum aestivum* L. and *Fagopyrum esculentum* Moench**.**	Aqueous extract of whole plant reduced the germination of test plants	Nazir *et al*., 2007
Rhynchosia capitata (Roth) DC.	*Vigna radiata* (L.) R. Wilczek	All the extracts affected germination and seedling growth, but higher inhibition was seen with leaf water extracts. Soil incoporated residues at higher concentration significantly reduced the seedling vigour index in addition to their significant effect on total germination	Ali *et al*., 2013
Rumex obtusifolius L.	Graminoids (non-leguminuous forbs and leguminuous forbs)	Species-specific susceptibility of grassland species	Zaller, 2006
Salvia plebia R. Brown	*Zea mays* var. 30-25 Hybrid, *Triticum aestivum* var. Pirsabak-04 and *Sorghum bicolor* L	Aqueous extract strongly affected the germination, plumule growth, radical growth, chlorophyll content and fresh and dry weights	Husna *et al*., 2016
Schima superba Gardner & Champ	*Phoebe bournei* (Hemsley) Yang	Aqueous extracts of leaf and root inhibited the germination rate, fresh weight and dry weight	XiaoQing *et al.*, 2006
Solidago canadensis L.	*Kummerowia striata* (Thunb.) Schindl	Allelopathic and competitive effects were greater in the introduced range and that allelopathy	Yuan *et al*., 2012

		significantly contributes to increased competitiveness for this invasive species	
Sonchus oleraceus L.	*Trifolium alexandrinum* L., *Brassica nigra* (L.) W.D.J. Koch, *Chenopodium murale* L., *Melilotus indicus* (L.) All. and *Sonchus oleraceus* L.	Lowest concentration of the plant extract partially inhibited germination and seedling growth whereas the higher concentrations inhibited those parameters completely	Gomaa *et al*., 2014
Tagetes minuta L. and *Eupatorium rugosum* Houtt.	*Aster scaber* Thunb., *Bidens bipinnata* L. and *Lotus corniculatus* var. *japonicus* Regel	Aqueous extract of whole plant inhibited the root and shoot length	Jihyon and KewCheol, 2006
Tinospora cordifolia (Willd.) Miers.	*Chenopodium album* L., *Chenopodium murale* L., *Cassia tora* L. and *Cassia sophera* L.	Aqueous extracts from root and aerial root significantly inhibited not only germination and seedling growth but also reduced dry weight	Raoof and Siddiqui, 2012b
Trifolium repens L.	*Abutilon theophrasti* Medic. and *Echinochloa crus-galli* (L.) P. Beauv	Aqueous extracts of aerial parts and roots reduced the seed germination, root activity, respiratory rate and enzyme activities	Ying *et al*., 2006
Trigonella foenum-graecum L.	*Lactuca sativa* L.	Physiological and biochemical changes were registered as a result of aqueous extract application	Omezzine *et al*., 2014a
Trianthema portulacastrum L., *Dactyloctenium aegyptium* L. and *Eleusine indica* L.	*Oryza sativa* L.	Germination and seedling growth showed differential behavior upon exposure to leaf, stem, root and seed water extract	Mubeen *et al*., 2011
Xanthium italicum Moretti	*Triticum aestivum* L.	Aqueous, organic and residue incorporation not only reduced seedling growth but also affected photosynthetic pigment content and photosynthetic parameters	Shao *et al*., 2013a

2.5. ALLELOCHEMICALS

The phenomenon of allelopathy is mediated by chemicals known as allelochemicals or allelochemics (Whittaker and Feeny, 1971). Whittaker and Feeny (1971) coined the term allele-chemicals; secondary metabolites sometimes act as allele-chemicals. Allelochemicals behave differently for expressing the growth behavior of the donor species, i.e retardatory effect at certain concentration of some species may stimulate the growth of same or different species at lower concentration (Narwal, 1994). Allelopathy is relatively a new branch of science (Lal and Oudhia, 1999). The various types of interactions (weed-crop, crop-crop, crop-weed and weed-weed interaction) can easily be explained with the aid of allelopathy. The allelochemicals present in flowers, leaves, leaf litter, leaf mulch, stems, bark, roots, soil, soil leachates and their derived compounds show variability in their activity and concentration over the growing season and with different plant parts (Kato-Noguchi, 2000; Qasem and Foy, 2001; Macias *et al.*, 2007; Jilani *et al*., 2008; Uniyal and Chhetri, 2010; Gatti *et al*., 2010). Under specific conditions, these chemicals make their way into the environment (atmosphere or rhizosphere) by means:

- Volatilization (Petrova, 1977; Oleszek, 1987; Bertin *et al*., 2003),
- Leaching (Bertin *et al*., 2003),
- Decomposition of residues (Guenzi *et al*., 1967; Hedge and Miller, 1990; Weston, 1996; Kohli *et al*., 2001),
- Root exudation (Tang and Young, 1982; Chou, 1990ab; Bertin *et al*., 2003),
- Pollen of some crop plants (Cruz-Ortega *et al*., 1988) and
- Exposure to stress conditions, extreme temperature, drought and UV exposure (Rice, 1984; Pramanik *et al*., 2000; Inderjit and Weston, 2003).

On the basis of structural variability and properties of these compounds (Li *et al*., 2010), allelochemicals can be classified into the following categories: (1) water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes and ketones (2) simple unsaturated lactones (3) long-chain fatty acids and polyacetylenes (4) quinines (benzoquinone, anthraquinone and complex quinines) (5) phenolics (6) cinnamic acid and its derivatives (7) coumarins (8) flavonoids (9) tannins (10) steroids and terpenoids (sesquiterpene lactones, diterpenes and triterpenoids). The biosynthetic pathways of the major allelopathic substances are shown in Plate 3 (Wang *et al*., 2006). The quantity of allelochemicals produced shows its dependence

on a variety of environmental conditions (Kobayashi, 2004; Bezuidenhout, 2005) such as

- Light: Some allelochemicals are influenced by the amount, intensity and duration of light. During exposure to ultraviolet and long-day photoperiods, the greatest quantities are produced. This is the reason for less allelochemicals present in under-storey plants because over-storey plants filter out the ultraviolet rays. At the peak plant-growing period, it could be expected that more allelochemicals are produced than earlier or later in the growing season (Ali, 2008)
- Mineral deficiency: Mineral deficiency paves way for the more production of allelochemicals.
- Drought stress: Production of allelochemicals is enhanced under these conditions.
- Temperature: Greater quantities are produced in cooler temperatures. The location within the plant and effects in specific allelochemicals seem to be variable (Ali, 2008).
- The type and age of plant tissue: Allelochemical production varies between species as well as within species. Therefore, it deserves its importance during extraction due to non-uniform distribution of compounds in plants.
- Shade, plant diseases and herbicides can influence allelopathy.

Allelochemicals (or, more in general, phytotoxins) are gaining more and more attention due to the current worldwide demand for cheaper, more environmental friendly weed management technologies. Thus allelopathy is a new branch of herbicide development (Macias *et al.,* 2007), because these compounds can be directly used as herbicide or may provide lead structures for herbicidal discovery (Duke *et al.,* 2000b). An important feature making allelochemicals good candidates as herbicides, is their selectivity towards some species, especially against weeds (Weston, 1996; Rimando *et al*., 2001; Ratnadass *et al*., 2012). Allelochemicals that suppress or eliminate competing plant species have received special attention (Macias *et al*., 2008a,b; Dayan *et al*., 2009) and many secondary metabolites with different molecular structures showing phytotoxic activity have been isolated and characterized from various sources (Fiorentino *et al*., 2006a,b,c, 2007, 2008; Scognamiglio *et al.*, 2012a,b).

Retention, transportation and/or transformation are the processes, which the allelochemicals undergo on entering into the soil (Cheng, 1995). For the toxicity of inactive compounds of donor plants in its immediate vicinity, degradation and transformation plays an important role (Rice, 1984). Besides, for the degradation and transformation of chemical compounds in the soil, various abiotic and biotic factors are responsible. Physical and chemical factors such as heat, light, soil texture, soil inorganic component and organic matter mainly include abiotic factors (Dalton *et al.*, 1983; Blum *et al.*, 1987; Dao, 1987; Inderjit and Dakshini, 1996; Einhellig, 1995) and microbes such as bacteria and fungi responsible for degradation/transport of organic molecules mainly in soil constitutes the biotic components (Rice, 1984; Blum and Shafer, 1988).

Different allelochemicals have been identified from different weeds by many workers. Perez and Ormeno-Nunez (1991) identified scopoletin, coumarin, *p*-hydroxy benzoic acid and vanillic acid as major allelochemicals of *Avena fatua* L. α–thujone identified from *Artemisia vulgaris* L. as major allelochemical by Dung *et al.* (1992). Inoue *et al.* (1992) identified emodin and physcion from *Polygonum sachalinense* F. Schmidt. The identification of phenolic acids viz. ferulic acid, vanillic acid, *p*-coumaric acid and *p*-hydroxybenzoic acids from *Sasa cernua* Makino (Li *et al.*, 1992). Chlorogenic acid identified from *Chenopodium album* L. by Mallik *et al*. (1994). Thus, there is a long list of allelochemicals. Table 4 shows some of the potential identified allelochemicals from weeds (Reports from 2005).

Table 4: List of different weeds with different allelochemicals identified (Reports from 2005).

Source	Chemical nature	Chemical Name	Reference
Ageratum conyzoides L.	Phenolic acids	*p*-coumaric acid, gallic acid, ferulic acid, *p*-hydroxybenzoic acid, comelic acid, anisic acid and protocatechuic acid	Batish *et al*., 2009a,b
Ambrosia trifida L.	Carotane-type sesquiterpenes	1α-angeloyloxy carotol and 1α-(2-methylbutyroyloxy)-carotol	Kong *et al*., 2007
Angelica sinensis (Oliv.) Diels	Monoterpenoid	4,6-dimethylbicyclo[3.1.0] hex-2-ene-2,6-dimethanol	Zhu *et al*., 2013

Artemisia sieberi Besser., *Artemisia judaica* L. and *Origanum dayi* L.	Volatile allelochemicals	1,8-cineole, camphor, borneol germacrene D, artemisia alcohol, trans-thujone, para-cymene, samphene, sabinene, *α*-pinene, pinocarvone, benzoic acid (methyl vanillate), cis-sabinene hydrate, *trans*-sabinene hydrate, myrtenol, carvacrol, jasmine ketolactone, (Z)-methyl jasmonate, *α*-tujene, eugenol, *cis*-thujone, terpinen-4-o, artemisia ketone, (E) ethyl cinnamate, davanone, artemisia alcohol, filifolide A, (Z) ethyl cinnamate, piperitone, *β*-davanone-2-ol, chrysanthenone, nor-davanone, borneol, yomogi alcohol, camphor, methyl vanillate, sabinene, jasmine ketolactone, methyl epi-jasmonate and methyl jasmonate	Friedjung *et al*., 2013
Avena fatua L.	Phenolic acids	*p*-coumaric acid, syringaldehyde and vanillin	Fragasso *et al*., 2012
Avena fatua L.	Phenolic acids	Syringic acid, vanillin, 4-hydroxybenzoic acid, syringaldehyde, ferulic acid, *p*-cumaric acid and vanillic acid	Iannucci *et al*., 2013
Avena fatua L.	Phenolic acids	Syringic acid, tricin, acacetin, syringoside and diosmetin	Liu *et al.*, 2016
Bletilla striata (Thunb.) Rchb. F.	Glycosidic compounds	Militarine and dactylorhin A	Sakuno *et al*., 2010
Cachrys pungens Jan	Flavonoids and phenolic acids	Naringin, quercetin, catechin, caffeic acid, ferulic acid and gallic acid	Araniti *et al.*, 2014
Calamintha nepeta L. (Savi)	Phenolic acids	Gallic, vanillic, syringic, *p*-coumaric and ferulic acids	Araniti *et al*., 2013

Carduus nutans L. and *Carduus acanthoides* L.	Taraxasterol, sitosterol, flavonoids	Kaempferol, apigenin, rutin and aplotaxene	Cerdeira *et al*., 2013
Centaurea diffusa Lam.	Sesquiterpene, fatty acid derived compounds	Caryophyllene oxide, linoleic acid, germacrene B and aplotaxene	Quintana *et al*., 2009
Centaurea diffuse Lam.	Quinoline	8-Hydroxyquinoline	Inderjit *et al*., 2010
Chenopodium album L.	Phenolic acids	Gallic, chlorogenic, caffeic, vanillic, *p*-coumaric, syringic and ferulic acid	Batish *et al.*, 2006a
Chenopodium murale L.	Phenolic acids	Vanillic, *p* hydroxybenzoic, cinnamic, caffeic, protocatechuic, ferulic and *p*-coumaric acids	Ghareib *et al.*, 2010
Chenopodium murale L.	Phenolic acids	Ferulic acid, vanillic acid, *p*-coumaric acid and benzoic acid	Batish *et al*., 2007a
Cleome arabica L.	Sterol, flavonol and damarane type triterpene	*β*-Sitosterol, 17-α hydroxycabraleactone, amblyone, calycopterin and 11-α-acetylbrachy-carpone-22(23)-ene	Ladhari *et al*., 2013
Cleome viscosa L.	Lactam	Nonanic acid LNA ((2-amino-9-(4-oxoazetidin-2-yl)-nonanoic acid)	Jana and Biswas, 2011
Diplostephium foliosissimum S.F. Blake	Hydroxycoumarin	Umbelliferone	Morikawa *et al*., 2011
Eclipta alba (L.) Hassk.	Phenolic acids	Vanillic acid, benzoic acid, ferulic acid and *p*-coumaric acid	Gulzar and siddiqui, 2015
Echinochloa crus-galli (L.) P. Beauv	Phenolic acids	*p*-hydroxybenzaldehyde, *p*-hydroxybenzen and *p*-hydroxybenzoic acid	Xuan *et al*., 2006
Echinochloa crus-galli (L.) P. Beauv	Phenolic acid	*p*-hydroxybenzoic acid	Esmaeili *et al*., 2012a
Echinochloa colona (L.) Link.	Flavonoid	Tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone)	Gomaa and AbdElgawad, 2012
Euphorbia esula L.	Diterpenes and jatrophane diterpenes	Kansuinine B and esulone A	Qin *et al*., 2006
Euphorbia dracunculoides Lam.	Phenolic acids	Furoic, *p*-coumaric, syringic and caffeic acids	Tanveer *et al*., 2012

Fagopyrum esculentum Moench.	Flavonoid and phenolic acids	Rutin, quercetin, (+) catechin, (-)-epicatechin, chlorogenic, caffeic, ferulic and gallic acids	Golisz *et al*., 2007
Fagopyrum esculentum Moench.	Phenolic acids and fatty acids	Palmitic acid, squalene, epicatechin, vitexin, gallic acid derivative, quercetin derivative, palmitic acid methyl ester, vanillic acid, rutin and 4-hydroxyacetophenone	Kalinova *et al*., 2007
Geranium carolinianum L.	Allelochemical	Ethyl gallate	Fujii *et al*., 2011
Imperata cylindrica (L.) Beauv.	Megastigmatrienone	4-(2-butenylidene)-3,5,5-trimethyl-2-cyclohexen-1-one (also called tabanone)	Cerdeira *et al*., 2012
Imperata cylindrica (L.) Beauv.	Alkaloid and phenolic acids	Hexadecahydro-1 azachrysen-8-yl ester ($C_{23}H_{33}NO_4$), gallic acid, caffeic acid, salicylic acid, snapinic acid, benzoic acid, cinnamic acid, emodin, ferulicacid,4-hydroxyphenylacetic acid, cholorogenic acid and resorcinol	Hagan *et al*., 2013
Inula falconeri Hook. F.	Eudesmane-type sesquiterpenoids	3β-caffeoxyl-β1,8α-dihydroxyeudesm-4(15)-ene	Khan *et al*., 2010
Jasminum officinale L. f. var. *grandiflorum* L.	Secoiridoid glucoside	Oleuropein	Teerarak *et al*., 2010
Lolium perenne L., *Dactylis glomerata* L. and *Rumex acetosa* L.	Benzoxazolinones and phenolic acid	Benzoxazolin-2(3*H*)-one (BOA) and cinnamic acid (CA)	Hussain and Reigosa, 2011
Lotus ornithopodioides L.	Phenolic acids and ferulic acids	Gallic, caffeic, chlorogenic, sinapic, vanillic, syringic, *p*-coumaric, phytone, methyl hexadecanoate, ethyl hexadecanoate, methyl octadecanoate and 4-methyl-2-heptanone	Araniti *et al.*, 2014
Leonurus sibiricus L.	Flavonoids and flavonoidic compound	Quercetin-3-O-a-L rhamnopyranosyl-(16)-b-D-galactopyranosid, rutin,	Rolim de Almeida *et al.*, 2008

		hyperin and isoquercetrin	
Mikania micrantha Kunth	Sesquiterpenoids	Dihydromikanolide deoxymikanolide and 2,3-epoxy-1-hydroxy-4,9 germacradiene-12,8:15,6-diolide.	Shao *et al*., 2005
Melilotus officinalis (L.) Pall.	Phenolic acid	Coumarin	Wu *et al*., 2016
Pluchea dioscoridis (L.) DC.	Phenolic acids	Gallic acid, syringic acid, catechol, *p*-nitrophenol, 4-chlorophenol, 4-chloro-3-methylphenol, vanillic acid, rutin tri-hydrate and kaempferol	Fahmy *et al*., 2012
Parthenium hysterophorus L.	Phenolic acids	Gallic, caffeic, 4-hydroxy-3-methoxy benzoic, *p*-coumaric, *m*-coumaric acids, ferulic, vanillic, syringic and *m*-coumaric acids	Safdar *et al*., 2014
Peganum harmala L.	Alkaloids	Harmine and harmaline	Shao *et al*., 2013b
Phalaris aquatica L.	Phenolic acids	Gramine	Adams *et al*., 2010
Rehmannia glutinosa (Gaertn) Steud.	Phenolic acid	Lauric acid and 2,6-Ditertbutyl phenol	Ming-Dao *et al*., 2009
Rhynchosia capitata (Roth) de Candolle	Phenolic acids	Vanillic acid and 4-(hydroxymethyl) benzoic acid	Ali *et al*., 2013
Sonchus oleraceus L.	Phenolic acids	Phenols, catechol, caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, resorcinol, sinapic acid, vanillic acid, flavonoids, catechin, rutin and quercetin	Gomma *et al*., 2014
Solidago canadensis L.	Allelochemical	n-hexadecanoic acid	DongYu *et al*., 2014

2.6. PHENOLIC ALLELOCHEMICALS

The class of most important and common plant allelochemicals in the ecosystem constitutes phenolic compounds (Li *et al*., 2010). Structurally, they consist of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group (Li *et*

al., 2010; Uniyal and Chhetri, 2010). Simple aromatic phenols, hydroxy and substituted benzoic acids and aldehydes, hydroxy and substituted cinnamic acids, coumarins, tannins and perhaps a few of the flavonoids represent phenolics (Zeng *et al*., 2008).

The most significant characteristics of phenolic compounds are in their structural diversity and intraspecific variability (Hartmann, 1996). Highly regulated processes of cell, tissue, development and environment-specific controls are required for biosynthesis and accumulation of phenolic compounds (Li *et al*., 2010). The pathways for phenolic compounds have been selected over time, among specific plant lineages, especially when these compounds take over specific advantageous functions (Li *et al*., 2010). In the shikimic and acetic acid (polyketide) metabolic pathways, 4-phosphate erythrose and phosphoenolpyruvic acid undergo condensation reactions with 7-phosphate altoheptulose, for phenolic compound formed after a series of transformation steps (Li *et al*., 2010) [Plate 3]. Besides, phenolic compounds are mainly held responsible for plant allelopathy by several authors (Li *et al*., 2010). Phenolic acids form the largest group of plant phenolics. Phenolic acids vary in their quantity and composition in the plants (Martens, 2002a,b). Vanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid (benzoic acid derivatives), caffeic acid, *p*-coumaric acid, sinapic acid and ferulic acid (cinnamic acid derivatives) constitute phenolic acids mainly identified in extracts from plant tissues.

Treatment with many phenolics express their impact on physiological parameters has commonly been observed (Plate 4). However, the mode of action of a chemical could broadly be divided into a direct and an indirect action (Blum, 2002). Indirect action includes the alternation of soil properties, nutritional status and an altered population or activity of microorganisms and nematodes.The allelochemicals with their biochemical/physiological effects on various important processes of plant growth and development represent direct action. Processes influenced by allelochemicals involve:

• Mineral uptake: Favorable rate at which ion absorption by plants takes place are altered by allelochemicals. In the presence of phenolic acids, both macro and micronutrients reduction occurred (Akemo *et al*., 2000).

• Cytology and ultrastructure: Inhibition of mitosis in plant roots occurred upon exposure to variety of allelochemicals (Mohamadi and Rajaie, 2009).

• Phytohormones and balance: Cell enlargement in plants is regulated by hormones indole acetic acid (IAA) and gibberellins (GA). IAA is present in both active and inactive forms and is inactivated by the IAA-oxidase. Various allelochemicals inhibited IAA-oxidase (Li *et al*., 2010) along with the effect on induced extension growth by other inhibitors.

• Membranes and membrane permeability: Changes in membrane permeability exerted by allelopathic nature of biological compounds. Exudation of compounds from roots on root slices have been used as an index of permeability because plant membranes are difficult to study (Gniazowska and Bagatek, 2005).

• Photosynthesis: Electron inhibitors or uncouplers, energy-transfer inhibitors, electron acceptors or a combination of the above may be the inhibitors of photosynthesis (Batish *et al*., 2001).

• Respiration: Stimulation or inhibition of respiration upon exposure to allelochemicals, both of which can be harmful to the energy-producing process (Batish *et al*., 2001).

• Protein synthesis: Allelochemicals inhibit protein synthesis as revealed by studies utilizing radio-labelled C P^{14} P sugars or amino acids and traced incorporation of the label into protein (Bertin *et al*., 2007; Batish *et al*., 2009b).

• Specific enzyme activity: Enzyme function in the plant (Muscolo *et al*., 2001) inhibits upon the treatment or exposure to a number of allelochemicals (Rice, 1984).

• Water relations (Sheteawi and Tawfik, 2007).

• Genetic material (Jensen *et al*., 2001).

• Growth and development: The growth and development of plants get affected upon exposure to allelochemicals. Inhibited or retarded germination rate, seeds darkened and swollen, reduced root or radicle and shoot or coleoptile extension, swelling or necrosis of root tips, curling of the root axis, discolouration, lack of root hairs, increased number of seminal roots, reduced dry weight accumulation and lowered reproductive capacity are however readily visible and distinguishable effects induced by allelochemicals (Wu *et al*., 1998). Acting at the cellular and molecular expression level in receiver plants, these gross morphological effects may be secondary manifestations of primary events, caused by a variety of more specific effects (Duke *et al*., 2000b). Seed germination inhibition upon exposure to allelochemicals appears to be caused by disruption of normal cellular metabolism rather than through damage

of organelles. Reserve mobilization, a process that usually takes place rapidly during the early stages of seed germination seems to be delayed or decreased under allelopathy stress conditions. Under allelopathic stress, reserve mobilization process for transport of organics during early stage of seed germination seems to be delayed or decreased (Gniazowska and Bagatek, 2005).

Multiple functions within a plant are being affected, although each mechanism of plant inhibition can lead to the reduced growth and/or death of an exposed plant due to mixture of allelochemicals from a donor species. Despite the extensive research with phenolic acids, target sites for allelochemical activity within affected plant species remain to be determined for many phenolic compounds. Although, phenolic acids are mainly known for their involment in allelopathy, also deserves their importance in chemical, biological, agricultural and medical studies. Recent interest in phenolic compounds stems from their potential protective role (i.e. through the ingestion of fruits and vegetables), against oxidative damage caused diseases, such as coronary heart disease, stroke and cancers. They are universally distributed in plants and plant decomposition products are their common source and they are important precursors of humic substances in soils. Free, reversibly bound and bound forms are three forms of phenolics found in soil. Chelate complexes with metals are formed by adsorption of ortho-substituted phenolics, such as salicylic and *o*-coumaric acids and dihydro-substituted phenolics, such as protocatechuic and caffeic acids by clay minerals. Soils flooded with vegetable wastewaters, especially accumulate free phenolic compounds in rhizosphere soils, besides altering the accumulation and availability of soil nutrients and rates of nutrient cycling, which both affect plant growth. The chemistry, biotechnology and ecotoxicology of naturally occurring polyphenols in vegetable waste have been reviewed by several studies (Capasso, 1997), phenolics protocatechuic acid and cathecol from onion are known for their protective role to aid against infection of *Colletotrichum circinaus* (Capasso *et al.*, 1992). Similarly, water-soluble phenolics inhibited spore germination and/or hyphal penetration of the pathogen by diffusion from the dead cell layers of the scales.

2.7. ALLELOCHEMICALS AND THEIR INTERACTION WITH SOIL NUTRIENTS

Upon release into the soil environment, phenolic forms component of the soil organic matter (Whitehead *et al.*, 1981, 1982; Inderjit, 1996; Martens, 2002b;

Kobayashi, 2004). Ferulic acid, *p*-coumaric acid, vanillic acid and protocatechuic acid are the most common phenolic acids found in soils (Whitehead *et al.,* 1982; Chou and Lee, 1991; Li *et al.,* 1992), *p*-hydroxybenzoic acid (Whitehead *et al.,* 1981; Kuiters and Dennemen, 1987), caffeic acid (Lodhi, 1976, 1978) and salicylic acid (Shindo *et al.,* 1978; Jalal and Read, 1983).

Various soil factors such as organic matter, inorganic ions, reactive mineral surfaces, ion-exchange capacity and biotic barriers alter the activity of allelochemicals in the soil (Inderjit, 2001). There is a need for soil processes to be considered in allelopathic research relevant to agricultural systems (Dalton, 1999) as the composition and quantity of allelochemicals modify over time with the changing environmental conditions (Dalton, 1999; Okumura *et al*., 1999). By ligand exchange reactions, soil surface catalyzed oxidation and/or incorporation into soil organic matter, phenolic acids react with soils abiotically (Dalton *et al*., 1989). Their subsequent oxidization and sorption by soil has been reported by (Lehmann *et al*., 1987; Makino *et al*., 1996). Organic matter and manganese oxide content in soil modify the phenolic acid sorption as found by Ohno and First (1998). The modification of allelochemicals by soil microorganisms due to their potential for degradation of toxic compounds or through the production of toxic compounds plays also an important role in allelopathy (Inderjit, 2001). In the soil-phenolic acid suspension, the increased concentration of Mn^{2+} provides the proof of phenolic acid oxidation upon sorption to the soil. Initial concentrations of allelochemical in soil influence its transformation pathway and degradation rate. The degradation and partial transformation of DIMBOA into MBOA occurs during ecotoxicological tests reported by Fomsgaard *et al.* (2006). Testing of MBOA on *Poecilus cupreusmedia* showed that at the initial concentrations of 2 and 10 mg kg^{-1} no MBOA was left after 45 days, but AMPO was formed and on both *Folsomia candida* and *Poecilus cupreus* test media BOA was transformed to a biologically more active compound APO. Along with microbial, non-microbial oxidative transformations by soil microorganisms plays an important role in allelopathic potential of *Polygonella myriophylla* (Weidenhamer and Romeo, 2004). AMPO and AAMPO and several novel compounds detected are the degradation products of MBOA and its isotopomer 6-trideuteriomethoxybenzoxazolin-2-one ([D3]-MBOA) in soil (Etzerodt *et al*., 2006).

Therefore, while studying the allelopathic potential of plant and various interactions occurring, the role of the soil should not be ignored (Inderjit *et al*., 2010).

Influence in soil pH, ammonia loss and wet nitrogen deposition reduction, with the alteration in chemical composition of rainfall reaching the soil occurs in grassland ecosystem upon accumulation of litter (Knapp and Seastedt, 1986; Facelli and Pickett, 1991). For the uptake of allelochemicals, soil pH has an important role (Blum, 1996). Amendment of soil by rice residues reduces the available nitrogen and other soil contents like Ca, Zn, Cu, Mn and Na (Chou and Chou, 1979). Polyvalent elements Cu^{+2}, Mn^{+2} and Fe^{+3} also facilitates the transformation of phenolic compounds stated by Pal *et al.* (1994). Nitrogen and phosphorus deficiency in barley (*Hordeum vulgare* L.) alerts allelochemicals *p*-coumaric and vanillic acids for expressing their effect on growth. In addition, allelochemicals also increase the solubility and mobility of metal ions when complexed with them such as protocatechuic acid complexed with Fe and Al (Shindo and Kuwatsuka, 1977a,b)

2.8. ALLELOPATHY IN RHIZOSPHERE SOIL

Although, above ground allelopathic interactions are well studied and characterized than interactions occurring in rhizosphere soil (Inderjit *et al*., 2007). However, allelochemicals produced from roots also express phytotoxicity through reduction in neighbouring plant growth and resistance or suppression of plant pathogens, soil microbes and other herbivores (Weston *et al*., 2012). The production and metabolism in the rhizosphere soil of bioactive secondary products in minute quantities with their release in soil needs to be characterized (Mohney *et al*., 2009). Specific allelochemicals in larger quantities and many different constituents in exudates varies among plant species (Watt and Weston, 2009). Exudation of allelochemicals from roots occurs by a passive leaching process. In the rhizosphere soil, secondary metabolites are synthesized, accumulated and actively secreted (Bertin *et al*., 2003; Bais *et al*., 2004; Prithiviraj *et al*., 2007ab). Identification of a number of phytotoxic compounds in plant root exudates deserves the mention of rhizosphere soil as the main site for allelopathic interactions (Kruse *et al*., 2000). Biomolecules released in rhizosphere from leachates, exudates or decomposition products and has been widely used in allelopathic studies (Bertin *et al*., 2003; Bais *et al*., 2004). Under field conditions, the growth inhibition of weed by root exudated allelochemicals has proven to be of ecological significance, however, their level needs to be sufficient for

adequate weed control or non-availability of plant material commercially (Perez and Ormeno-Nunez, 1991; Olofsdotter *et al.,* 1999; Fujii, 2001)

The organisms present in rhizosphere soil serve numerous functions, among them include

- Production and utilization of potential allelochemicals by decomposition (mineralization and humification) of organic residues enhancing plant growth through production of plant hormones.
- Increase the availability of nutrients.
- Enhancement of nutrient use efficiency.
- Provide protection against root pathogens and possibly phytotoxins.

2.9. ALLELOPATHIC INTERACTION BETWEEN WEED AND CROP

The chemicals generated by allelopathic plants rely their impact on nearby plants making a way for new discovery to manage weed-crop allelopathy. The concept implies the use of crop cultivars with a built in herbicidal system capable of producing and releasing sufficient amounts of phytotoxic allelochemicals via root exudation that interfere with competing weed efficiently.

A crop with allelopathic potential should include the following characteristics:

(i) Affect the growth, productivity and yield of other crop.

(ii) May affect same crop growing in monoculture or grown in succession.

(iii) Cause soil sickness and imbalance of nutrients and microbial population and

(iv) Can be exploited to selectively suppress weeds through various manipulations (Einhellig, 1985a; Batish *et al.*, 2001).

The allelopathic potential of 1700 rice accession was evaluated, out of these 557 inhibited the growth of *Heteranthera limosa* and *Ammannia coccinea* in a field experiment conducted (Dilday *et al.,* 1994, 1998). It is uncertain, whether these inhibitions were caused due to the allelopathic interference or there might be some other competitive influence because interaction between plants is a complex combination of interferences for resources and hence allelopathy cannot be separated under field condition (Olofsdotter *et al.,* 1999).

Aqueous extract of rice plant (Kawaguchi *et al*., 1997) inhibits growth of several plant species and aqueous extracts of decomposing rice residue inhibited root growth of lettuce seedling (Chou and Lin, 1976). Identification of several phenolic compounds, such as *p*-hydroxybenzoic acid, vanillic acis, *p*-caumaric acid and ferulic

acids were noticed in aqueous extracts of rice residue and straw (Chou and Chou, 1979). Separating allelopathic effect from other interference mechanisms needs a clear concept for understanding its relative importance rather than simply its existence as revealed by several workers (Nilsson, 1994; Weidenhamer, 1996; Ridenaur and Callaway, 2001). Allelopathy and resource competition can be separated based on phytotoxic effects, according to Weidenhamer *et al.* (1989). Interference among plants by different mechanism may operate simultaneously or sequentially in nature as depicted clearly (Inderjit and Moral, 1997). Statistical measurement of treatments in contrast to the control is needed as stated by Williamsom and Richardson (1988). In allelopathic bioassays, the most widely used parameter is seed germination (Rasmussen and Einhellig, 1977; Stowe, 1979; Williams and Hoagland, 1982; Rice, 1984). However, some workers found the oven dry weight of radicle (Leather and Einhellig, 1985) and root length and root fresh weight to be statistically more accurate (Cope, 1982; Pederson, 1986). Phytotoxic effect of quack grass (*Agropyron repens* (L.) Beauv) against germination and radicle growth of several species of weed and crop plants was reported due to its aqueous extract (Weston *et al.*, 1987).

Phenolic compounds in combination interact synergistically because the soil concentrations (usually<1 Mm) of the phenolic compounds do not reduce germination significantly (Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978). During decomposition of crop residues, the germination and growth of others get adversely affected. The allelochemicals released from preceding crops affect the performance of succeeding crops. In sugar beet, however, a number of soluble phytotoxic allelochemicals released by the residue accumulation in soil and the crops roots may have a chance encounter with these chemicals leading to serious repercussion on the quality and quantity of crop yields (Kalburtji and Gagianas, 1997).

The allelopathic property of a number of crops on other crops has also been noticed in succession or simultaneously or may even exhibit autotoxicity (Einhellig, 1985a,b; Putnam and Weston, 1986; Anaya, 1999; Chou, 1999; ChuiHua *et al.*, 2007). For the purpose of weed management, cover crops especially may be exploited (Weston, 1996; Foley, 1999). Old roots remaining hidden in soil after harvest of crops are the principal cause of crop autotoxicity through the release of phytotoxins that affect the succeeding crops directly, cause microbial imbalance, change in organic

matter of soil, increase ion leakage, disturb nutrient uptake and immobilization (Katznelson, 1972; Kimber, 1973; Yu and Matsui, 1997). Rice (Chou, 1995), wheat (Kimber, 1973), maize (Yakle and Cruse, 1983, 1984), sugarcane (Chou, 1995) and several vegetable crops like cucumber, carrot, funnel, watermelon, egg plant, tomato and even pea (Yu, 1999) are among the highly worked out crops exhibiting autotoxicity. Some allelochemicals (mainly phenolic acids) identified from weed residue are listed below in Table 5.

Table 5: Allelochemicals identified from weed residue.

Common name	**Botanical name**	**Allelochemicals (s)**	**Reference(s)**
Buckwheat	*Fagopyrum esculentum* Moench	Ferulic, caffeic, chlorogenic, palmitic, stearic, arachidic and behenic acids	Tsuzuki and Dong, 2003
Silver grass	*Vulpia* spp.	Pyragallol, catechol, 3, 4-dimethoxyphenol, coniferyl alcohol, vanillic, *p*-coumaric, hydroquinone, protocatechuic, benzoic, *p*-hydroxy-benzoic, hydrocinnamic salicylic, gentisic, syringic, succinic, α-hydroxy-benzenepropanoic, *p*-hydroxybenzene propanoic, hydrocaffeic, *p*-hydroxyphenyl acetic, hydroferulic and ferulic acids	An *et al.*, 2000a,b
Nettle leaved goosefoot	*Chenopodium murale* L.	Protocatechuic, ferulic, *p*-coumaric and syringic acid	Batish *et al*., 2007b
Bill goat weed	*Ageratum conyzoides* L.	*p*-coumaric acid, gallic acid, ferulic acid, *p*-hydroxybenzoic acid and anisic acid	Batish *et al*., 2009b
Bill goat weed	*Ageratum conyzoides* L.	Gallic acid, coumalic acid, protocatechuic acid, catechin and *p*-hydroxy benzoic acid	Batish *et al*., 2009a
Jerusalem artichoke	*Helianthus tuberosus* L.	Salicylic acid, *p* hydroxybenzaldehyde, cinnamic acid, *o*-coumarinic acid, *p*-coumarin acid and coumarin	Tesio *et al*., 2011
Bindweed	*Convolvulus arvensis* L.	Cinnamic acid, *p*-coumaric acid, coumarin, ferulic acid, syringic acid, salicylic acid, caffeic acid, resorcinol, pyrogallic acid, protocatechuic acid, chlorogenic acid and *p*-hydroxy benzoic acid	Hegab and Ghareib, 2010
Awnless barnyard grass	*Echinochloa colona* (L.) Link.	Coumarin, resorcinol, apigenin, cinnamic, syringic, chlorogenic, ferulic and protocatechuic acids	Gomaa and AbdElgawad, 2012

Bill goat weed	*Ageratum conyzoides* L.	Gallic, sinapic, coumaric, protocatechuic, *p*-coumaric, *p*-hydroxybenzoic and benzoic acids	Xuan *et al.*,2004
Lantana	*Lantana camara* L.	Vanillic, *p*-hydroxybenzoic, *p*-coumaric, protocatechic, gentisic, caffeic, syringic, ferulic, *o*-coumaric, trans-cinnamic and salicylic acids	Achhireddy e*t al.*,1985; Ambika *et al.*, 2003
Rat's-tail fescue	*Vulpia myuros* (L.) C.C. Gmel.	Syringic, vanillic, succinic acids, catechol and hydrocinnamic acid	An *et al*., 2001
Common thistle	*Cirsium japonicum*	Coumarin, trans-cinnamic acid and chlorogenic acid	Chon, 2004
Siam weed	*Chromolaea odorata* (L.) King & Robins	Phenolic acids and alkaloids	Ambika, 1999
Wild red rice	*Oryza perrennis* Moench nom. dub.	Phenolic acids	Chou *et al.*, 1991
Yellow Fieldcress	*Rorippa sylvestris* (L.) Besser	Salicylic, *p*-hydroxybenzoic, vanillic, syringic acids, hirsutin and pyrocatechole isothiocyanates.	Yamane *et al.*, 1992
Lamb squarter	*Chenopodium album* L.	Chlorogenic acid	Mallik *et al.*, 1994
Arrow bamboo	*Sasa cernua* Makino	Ferulic, vanillic, *p*-coumaric and *p*-hydroxybenzoic acids.	Li *et al.*, 1992

2.10. RELATION OF FOLIAR MICROMORPHOLOGY TO ALLELOPATHY

Pharmaceuticals, neutricals, natural pesticides, flavouring and fragrances or even for non-food of fibre purposes desire their importance due to diverse biological activities and functions of secondary metabolites produced by plants (Duke *et al*., 2000a). Plants mainly produce and store these secondary compounds on or near plant surfaces and in certain cases these also take the shape of specialised cells called glandular trichomes in concentrated form for maximum effect when sequestered and also their interaction with the outside world. These specialised cells will protect the plant from autotoxicity and have various functions such as secretion. These secretory cells take the shape of trichomes (Fahn, 2000), glandular hairs, stinging hairs or the epidermis itself (Wink, 1999) and glands on the external surfaces of many plants. Specialised secretory glandular trichomes act either as a storage reservior or volatilization of secondary metabolites (allelochemicals) from the leaf surfaces occur.

Between gland cell walls and the cuticle outside the plant body the storage of metabolites occurs (Wagner, 1991).

Secondary metabolites are considered allelopathic by depressing the seed germination of other plant and there by increasing the competitiveness of the plant containing metabolites in the trichomes (Roshchina and Roshchina, 1993). The growth inhibiting metabolites contained in soft, fine trichomes of *Parthenium hysterophorus* L. caused allelopathic growth inhibition of ten day old wheat (*Triticum aestivum* L.) seedlings (Kanchan and Jayachandra, 1980). The allelochemical responsible for growth inhibition in roots and shoots of the test species lettuce (*Lactuca sativa* cutivar Nigra), tomato (*Lycopersicon esculentum* Mill.), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) noticed was sesquiterpene lactone found in glandular trichomes on the leaves of sunflower (*Helianthus annuus*) L. (Macias *et al*., 1996). The alkaloides with their allelopathic nature has been secreted from the glandular hairs of two solanaceous species viz. potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tobacum* L.) as per stated by Roshchina and Roshchina (1993). The secreting trichomes from the above-mentioned species as well possibly from *C. procera* accumulate allelochemicals in a significant way and the secreted substances can reach levels up to 10 to 30% of a plant's dry weight (Kelsey and Reynolds, 1984).

2.11. ALLELOPATHY AND CYTOTOXICITY

The allelochemicals from donor plants affect cell division, which results into positive or negative impact on growth of recipient plant. Hence, the screening of cytotoxic activities of such plants is necessary.

Table 6: Allelopathic weeds with cytogenetic effects on target plants (Reports from 2007).

Source	Target plant	Part and allelochemicals used	Reference(s)
Parthenium hysterophorus L.	*Allium cepa* L.	Parthenin	Batish *et al*., 2007c
Jasminum officinale L. f. var. *grandiflorum* (L.) Kob.	*Allium cepa* L.	Oleuropin	Teerarak *et al*., 2010
Vicia villosa ssp.*varia* Roth.	*Allium cepa* L.	Cyanamide	Soltys *et al*., 2011
Parthenium hysterophorus L.	*Vicia faba* L.	Parthenin	Raoof and Siddiqui,

			2013b
Brassica nigra L.	*Pisum sativum* L.	Aqueous extract	Mohamed and El-Ashry, 2012
Lantana camara L.	*Lathyrus sativus* L.	Leaf aqueous extract	Talukdar, 2013
Parthenium hysterophorus L.	*Helianthus annuus* L.	Decomposed leaf extract	Kumar and Gautam, 2008
Fimbristylis miliacea (L.) Vahl	*Allium cepa* L.	Aqueous extract	Siddique and Ismail, 2013
Origanum vulgare ssp. *vulgare* L.	*Allium cepa* L.	Cold water extract of aerial parts	Dragoeva *et al*., 2014
Cassia occidentalis L.	*Allium cepa* L.	Aqueous extracts of root, leaf, flower and stem	Arora, 2013
Lantana camara L.	*Vigna mungo* L. Var. *Vamban*-16	Aqueous extracts of root,stem, leaf, flower and fruit	Rajendiran *et al*., 2014
Conyza bonariensis (L.) Cronquist	*Vicia faba* L. and *Zea mays* L.	Aqueous extracts of leaf, fruitlet, stem and root	LiJuan *et al*., 2013
Rhazya stricta Decne.	*Vicia faba* L.	Extracts from fresh leaves	Mutawakil, 2012
Cymbopogon citratus (DC) Stapf	*Lactuca sativa* L.	Aqueous extracts of leaves	Sousa *et al*., 2010
Parthenium hysterophorus L.	*Helianthus annuus* L.	Leachates of leaf and inflorescence	Mohanan and Rajendiran, 2014
Trifolium repens L.	*Vicia faba* L.	Aqueous extract	Yang-Yan *et al*., 2010
Datura stramonium L.	*Glycine max* (L.) Merrill.	Aqueous leaf extract	Cai and Mu, 2012
Chromolaena odorata (L.) King and Robinson	*Lathyrus sativus* L. and *Lens esculenta* Moench.	Leaf leachates	Nandi and Mandal, 2010
Mirabilis jalapa L.	*Vicia faba* L.	Aqueous extract	Xiao-Kui *et al*., 2008
Hyssopus officinalis L.	*Allium cepa* L.	Cold and hot infusion	Dragoeva *et al*., 2010

Chapter-3
Materials and Methods

MATERIALS AND METHODS

For the present investigation, different types of materials of *Calotropis procera* (Ait.) R. Br. and soil under it were collected from areas in and around Aligarh Muslim University, Aligarh, where the weed grew predominant.

3.1. PROCUREMENT OF SEEDS

The viable, healthy and uniform seeds of crop plants (*Triticum aestivum* L., *Pisum sativum* L., *Brassica oleracea* var. *botrytis* L. and *Spinacea oleracea* L.) and weed plants (*Cassia sophera* L., *Cassia tora* L., *Cannabis sativa* L. and *Chenopodium album* L.) were procured from the Indian Agriculture Research Institute, New Delhi and National Research Centre for Weed Science, Jabalpur (Madhya Pradesh), respectively.

3.2. COLLECTION OF PLANT MATERIAL

The plants of *C. procera* were collected locally from roadsides and other areas around the University campus. Different parts, i.e. green leaves, stem and roots were separated from the plant at flowering stage. Each part was separately dried, powdered and stored in labeled polyethylene bags till used.

3.3. COLLECTION OF RESIDUES

3.3.1. Root residue

The plants of *C. procera* were uprooted and roots were separated from them. These were a shade dried, powdered and stored in polyethylene bags for further use and referred to as *root residue.*

3.3.2. Above ground residue

Plant residue from mature stands (after seed setting stages) was manually harvested about one cm above the soil surface, collected, powdered and packed in polyethylene bags and referred to as *above ground residue.*

3.4. COLLECTION OF SOIL

3.4.1. Rhizosphere soil

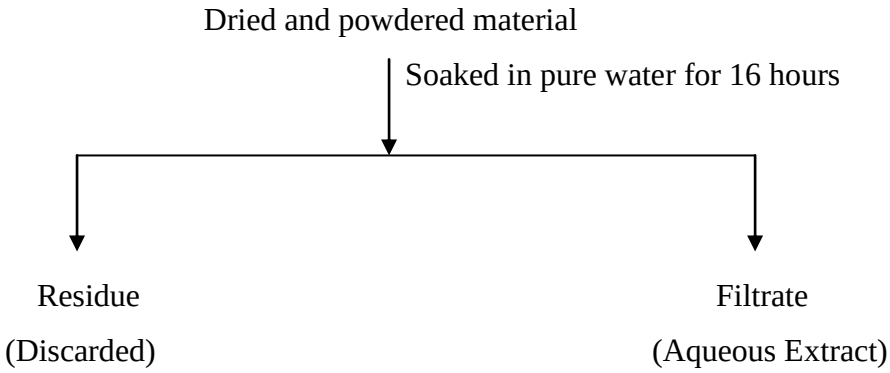
Rhizosphere soil, i.e. soil in and around the root system (approximately at 5-15 cm depth and 10 cm radius) was collected from *C. procera* invaded agricultural fields or other selected areas on the outskirts of Aligarh. The soil was collected from five different sites and each site from five areas. Collection of soil was made from the upper 0-15 cm soil profile since 80% of the root system of weed is present in this zone.

3.4.2. Control soil

The soil was also collected from nearby areas free of *C. procera* (at least 60 m away) to serve as controls. The collected soil samples were shade dried, sieved through a 2 mm sieve and filled in duly labeled polyethylene bags till further use.

3.5. PREPARATION OF C. PROCERA EXTRACTS

For this purpose, weighed amount of plant material of each type was immersed separately in the requisite amount of pure water (conductivity<0.05 at 25ºC) for 16 hours.



Protocol: Scheme for extraction of aqueous extract.

The content was filtered. The filtrate was termed as aqueous extract and stored in refrigerator for further use.

3.6. PREPARATION OF AMENDED SOILS

Amendments in soils were done in two ways. In the first case, dried and powdered plant material (aboveground parts, leaves or roots) was directly mixed with soil. In another case, aqueous extract prepared from plant material was added to the soil.

3.6.1. Powder amended soils

In this case, the requisite amount of dried powder (or residue) of plant material was mixed in soil so as to get concentrations of 0.5,1,2 and 4% (w/w). The contents were mixed well and used for growth studies. These were termed as *powdered amended soils.*

3.6.2. Extract amended soils

In this case, 500 ml of the extract of each concentration was added to 1 kg of soil. These were mixed well, dried and termed as *extract amended soil.*

3.7. GROWTH STUDIES

3.7.1. Under laboratory condition

3.7.1.1. Germination trials

The seed germination trial was performed following International Seed Testing Association rules. Uniform, healthy, viable seeds of the plant under test were procured. These were surface cleaned and subjected to germination trial in response to the treatments. For each treatment, seeds of each requisite type were taken and soaked in respective treatment solutions at room temperature for 24 hours. Treatment with pure water served as control. The seeds of each treatment were placed in 15 cm diameter Petri dishes. For this purpose, each Petri dish was lined with a thin absorbent cotton wad with Whatman No. 1 filter paper. The disc was moistened with 15 ml of aqueous extract or pure water (control)). Five replicates were maintained for each treatment. The whole set was placed in a seed germinator maintained at 30±2°C temperature, relative humidity of 25±1% and continuous light of approximately 4000 Lux for 24 hours daily. Everyday observations on the emergence of radicle were made with the help of a hand lens in one of the respective Petri dishes (out of five maintained for each treatment), observations were done continuously for 15 days till no more seeds germinated. Seed vigour (an index of speed of germination) was determined for seeds of representative Petri dishes in which daily counts on the number of seeds that germinated were made. For calculating the seed vigour percent, following formula as given by Agarwal (1980) was employed.

$$\text{Seed vigour} = \sum \frac{\text{Qutients of daily counts}}{\text{Number of days of germinatio n}} \times 100$$

After 15 days of germination, radicle length and plumule length of all germinated seedlings were measured and also determined their weight by drying in oven set at 75ºC for 20 hours.

3.7.1.2. Under greenhouse condition (In pots)

Growth studies were conducted in November, for winter season crops/weeds and in April for the summer season crops/weeds, in pots maintained at net house. For this purpose, 700 g of rhizosphere soil of *C. procera,* all types of amended soil as well as control soil (unamended) were filled in dully labeled plastic pots (12.5 cm diameter). Five replicates were maintained for each test plant. Seven healthy and viable seeds of each crop/weeds were placed at 1 cm below the soil surface in each pot. The pots were placed under natural conditions and arranged in a completely

randomized block design and adequately watered daily. The plants in each pot were allowed to grow for one month (in case of rhizosphere soil) or eight days (in case of other amended soil). After one week or month, the number of plants emerged, were counted. These were carefully uprooted and their root length, shoot length and dry weight was determined by oven drying at 75ºC for 48 hours.

3.8. DETERMINATION OF pH OF EXTRACTS

The pH of each extract prepared from different plant parts was determined by immersing the electrode of Eco Scan digital pH meter (Eutech Instruments, Singapore). It was presented as mean of five replicates.

3.9. DETERMINATION OF CONDUCTIVITY OF EXTRACTS

The conductivity of the plant extract was measured with the help of an Eco Scan Con 5 digital conductivity meter (Eutech Instruments, Singapore) by immersing its electrode into each extract. For this, five replicates were also maintained and expressed the mean values in µS or mS.

3.10. DETERMINATION OF OSMOTIC POTENTIAL

Osmotic potential of extracts of plant material was determined using the following formula:

Osmotic Potential = 0.36×Conductivity (mS)

3.11. DETERMINATION OF TOTAL PHENOLIC CONTENT IN EXTRACTS

Total phenolic content in aqueous extracts was determined spectrophotometrically using folin cio-calteu reagent as per the method of Swain and Hillis (1959). To 1 ml of extract was added 1 ml of folin-ciocalteu (50% diluted) reagent and 1 ml of 20% Na_2CO_3. It was allowed to stand for 30 min. till blue color developed. This blue color was read at 700 nm on spectrophotometer against known concentration of ferulic acid. Pure water to which same reagents were added to serve as a blank. For each test tube, five replicates were maintained. The amount of phenolic was expressed as µg/ml.

3.12. EXTRACTION OF LEACHABLE ALLELOCHEMICALS (PROTOCOL: I)

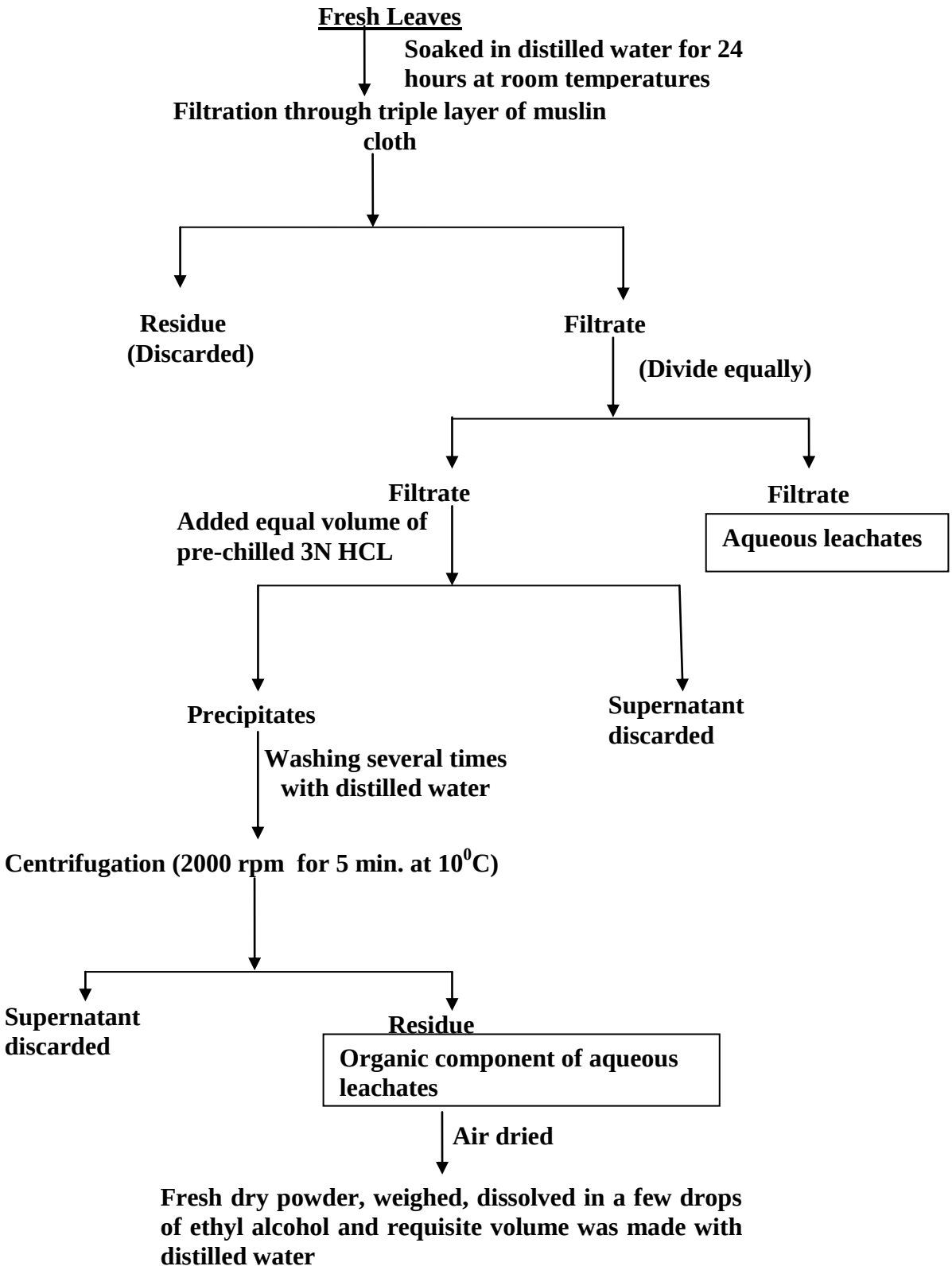
Based on the methods devised by Kumari *et al*., 1985, healthy and freshly collected leaves of *C. procera* were cut roughly into pieces after clearing their surface and their dry weight per unit fresh weight were determined by desiccating the tissue in the oven.

The weighed amount of fresh leaf pieces of the plant was soaked in the requisite amount of pure water (resistivity more than 18.5 mega ohms cm and conductivity less than 0.05µ Simons cm at 25°C) for a period of 20 hours at room temperature. It was filtered completely through a triple layer of muslin cloth and the requisite concentration was made with water. One half of this filtrate referred to as the aqueous leachates was used as such, while the other part was chilled and subjected to acid hydrolysis using pre-chilled, 3N HCl. The precipitate so formed were recovered through centrifugation (2000 rpm). These were washed 5-6 times with pure water. Every time the recovery was made through centrifugation. For experimental purpose, the requisite amount of the precipitate was dissolved in a few drops of ethyl alcohol and the final volume was made with pure water. A drop of tween 20 was added to it, to serve as a surfactant. This is referred to as aglycone or aglyconic or organic component of aqueous leachates. In this study, however, aglyconic components have not been taken into consideration due to the insignificant formation of the precipitates when 3N HCl was added.

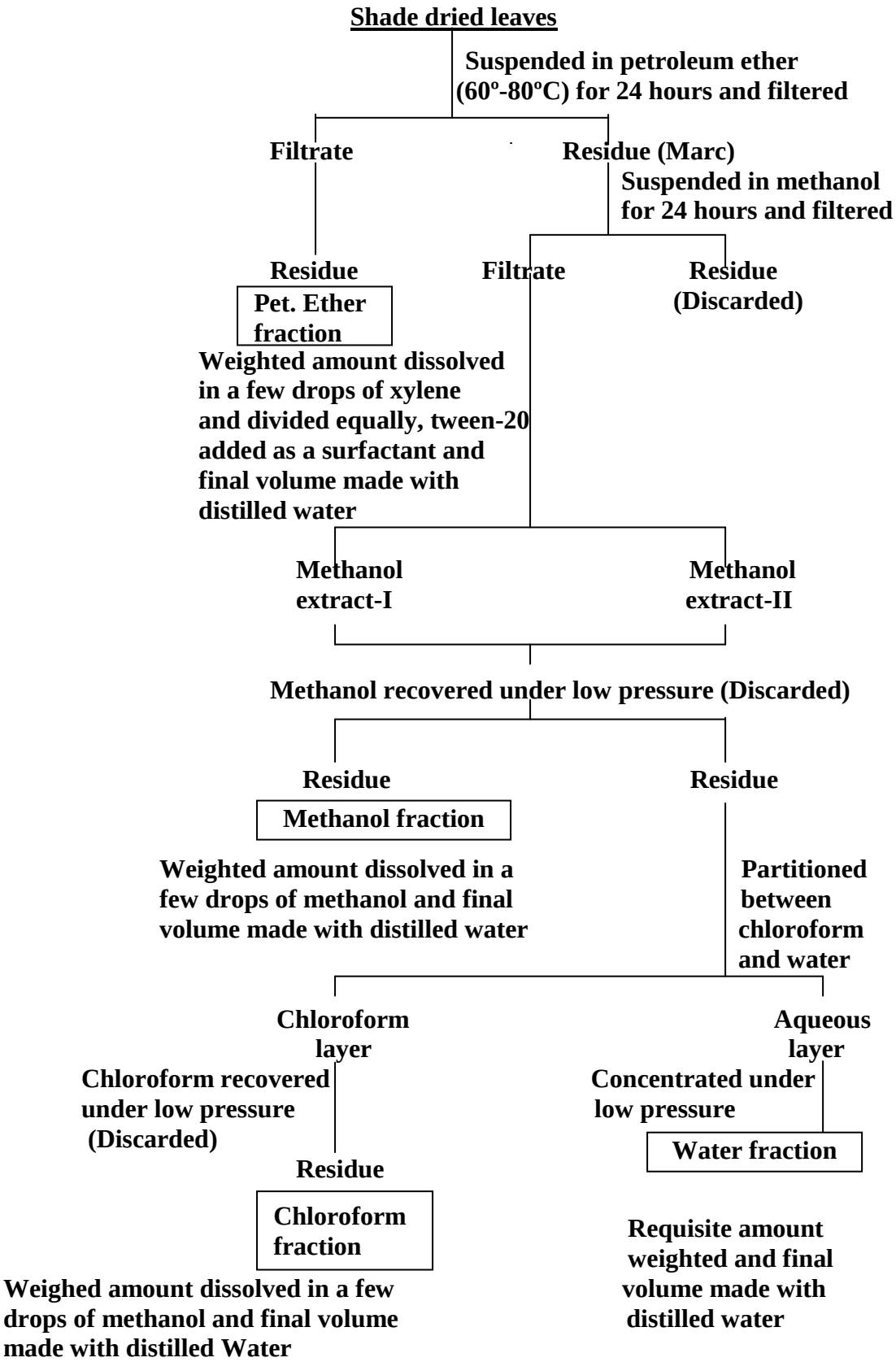
3.13. EXTRACTION OF ORGANIC FRACTION (PROTOCOL: II)

Freshly collected, surface cleaned and healthy leaves of the requisite plant were dried under shade and powdered. The powder was immersed in petroleum ether (60^0-80°C) for 20 hours. The liquid was separated from the residue (Marc), through mild centrifugation (500 rpm for 2 min.). From the liquid portion, the solvent (petroleum ether) was recovered on a hot water bath. The requisite amount of the residue so obtained was weighed and a few drops of xylene, apart from a drop of tween-20 (to act as a surfactant) were added to it. Final volume was made with pure water. This was termed petroleum ether fraction (PF).

The Marc (residue from petroleum ether suspension) was suspended in methanol for 20 hours and filtered, from one-half of the filtrate, methanol was recovered on a hot water bath. The residue, so obtained was dissolved in a drop of methanol and the final volume was made with pure water. It has been called methanol fraction (MF). From another half of methanol filtrate, the solvent was removed and the residue was partitioned between chloroform and water 1:1 (v/v). The two layers so formed were separated in a separating funnel. The chloroform was recovered over a



Protocol I: Extraction of aqueous leachates and organic component of aqueous leachates (After Kumari *et al.*, 1985).



Prorocol II: Extraction of different organic fractions from the leaves (After Kumari *et al.*, 1985).

hot water bath. To the requisite amount of residue, a few drops of methanol were added and the final volume was made with pure water. This has been termed as the chloroform fraction (CF). The water from the aqueous layer after separating chloroform fraction was dried under low pressure on a rotary flash evaporator. The solution made with water has been termed as the water fraction (WF).

3.14. CONTENT OF MACRO-MOLECULES

3.14.1. Preparation of dry material for macro-molecular estimation

3.14.1.1. Treatment to mature plants

The plants of the test species were raised in earthen pots of 6 inch diameter. The plants were given a fine mist of aerial spray (20 ml/plant) of the requisite concentration of requisite treatment solutions for three consecutive evenings.

The material for estimation of macro-molecules was crushed in acetone. It was freed of pigments by repeated washing in acetone for about three days. The crushed tissue was then put in 1:1 v/v mixture of acetone and petroleum ether for 24 hours, followed by further suspension in petroleum ether for 2 hours and then air dried. The dried powder was used for estimation of total proteins, water soluble and acid soluble carbohydrates.

3.14.1.2.. Estimation of total soluble proteins

The method as given by Lowry *et al.,* 1951 was adopted for this purpose.

Reagents

a- 2% sodium carbonate in 0.1 sodium hydroxide.

b- 0.5% copper sulphate in 1% sodium citrate.

c- 50 ml of reagent 'a' mixed with 1 ml of reagent 'b'.

d- Folin Cio-calteu reagent from BDH (diluted with two volumes of pure water before use).

Estimations

To 5 mg dry powder of the material was added 5 ml of reagent ‘c’ with simultaneous thorough shaking. After 10 min., 0.5 ml of reagent’s was added to it and the solution was shaken properly. The concentration of protein of the blue coloured solution thus obtained was read directly after half an hour at 700 nm against 0.1 mg/ml of standard albumin on dual beam supertonic 1201 spectrophotometer pressing the use of concentration key. The content was expressed as mg/g dry weight.

3.15. CARBOHYDRATE CONTENT

The methodology employed by Loweus, 1952 was followed for this purpose.

3.15.1. Extraction

(a) Water soluble carbohydrates-To 5 mg dry powdered material was added 5 ml of pure water. It was kept in boiling water bath for 5 min. and centrifuged. The supernatant was used as an acid soluble fraction (ASF).

(b) Acid soluble carbohydrates-To the residue left as above was added 5 ml of 6 N HCl. This was kept in a boiling water bath for 20 min. and centrifuged. The supernatant was used as water-soluble fraction (WSF).

3.15.2. Estimation

To 1 ml solution (each of WSF OR ASF) was added 4 ml of anthrone reagent (0.2% anthrone dissolved in concentrated H_2SO_4). The tubes were kept in boiling water bath for 10 min. The concentration of carbohydrates from the brownish yellow to green colour was read at 620 nm. By pressing concentration key in the dual beam spectronic 1201 spectrophotometer against a known concentration of glucose as standard. The carbohydrate content was expressed as mg/g dry weight of material.

3.16. ESTIMATION OF CHLOROPHYLL CONTENT

The total chlorophyll content from the leaves of treated or control plants were extracted in Di-methyl sulphoxide (DMSO) following the method of Hiscox and Israelstam, 1979. Finely cut uniform discs (100 mg fresh weight) were made from fully expanded leaves of test plants. Dry weight equivalents of each of the treated samples were determined by keeping 100 mg fresh weight discs in an oven.

The weighted material (100 mg fresh weight leaf disc) was suspended in 10 ml of Di-methyl sulphoxide (DMSO) incubated at 65°C for one hour (the period of incubation was found sufficient for the complete extraction of chlorophyll). The DMSO was recovered by thorough decantation. The final volume was corrected to 10 ml with fresh DMSO. The extinction of chlorophyll thus recovered in DMSO was measured at dual wavelength of 645 and 663 nm on spectrophotometer against DMSO as blank. The extinction values were read and the amount of chlorophyll was calculated according to the equation given by Arnon (1949), with modification by Hiscox and Israelstam (1979).

Total Chl. (µg/mg) = (6.45×A663) + (17.72×A645).

Where A645 and A663 represent extinction at values 645 nm and 663 nm, respectively.

3.17. SOIL ANALYSIS

3.17.1. DETERMINATION OF SOIL pH AND CONDUCTIVITY

Soil extracts were prepared by mixing dried soil and pure water in the ratio 1: 2 (w/v). For this, 20 g soil was mixed in 40 ml pure water. At least five samples, each were kept for both *Calotropis* and control soil. The slurry of each soil type was stirred thoroughly for 1 hour on electric shaker and kept undisturbed for 15 min. Aqueous extracts of respective soils were taken in beakers. The pH and conductivity of soil extracts were read directly with pH and conductivity meter, respectively.

3.17.2. ESTIMATION OF ORGANIC CARBON

Organic carbon was estimated by a rapid titration method of Walkley and Black, 1934.

Reagents

1. Potassium dichromate solution ($K_2Cr_2O_7$), 1N (normal) - Dissolved 49.04 g of $K_2Cr_2O_7$ (AR Grade) in 900 ml pure water and made the volume 1 litre.
2. Phosphoric acid-85%.
3. Concentrated sulphuric acid (H_2SO_4).
4. Diphenylamine-Dissolved 0.5 g diphenylamine in a mixture of 100 ml concentrated sulphuric acid and 20 ml pure water.
5. Ferrous sulphate, N/2-Dissolved 139 g of $FeSO_47H_2O$ (AR Grade) in water and added 15 ml of concentrated H_2SO_4 and diluted to 1 litre.

Procedure

Took 1 g of the soil sample into a 500 ml Erlenmeyer flask and added to it 10 ml of $K_2Cr_2O_7$ followed by 20 ml of concentrated H_2SO_4. Shook the contents of the flask for one min. and kept aside for half an hour. Then, added 250 ml of pure water and 10 ml of phosphoric acid and 3-4 drops of diphenylamine indicator solution. The contents of the flask turned blue. Titrated the content against ferrous sulphate solution until the colour changed to green.

Calculations

1 ml of 1N $K_2Cr_2O_7$ is equivalent of 3 mg of carbon. The amount of carbon oxidized, expressed as percentage of soil, is given by

$$\% \text{ Organic Carbon in soil} = \frac{\text{Titre Value (ml)} \times 0.003 \times 100}{\text{Weight of the soil taken (g)}}$$

Where titre value = Total volume (ml) of 1N $K_2Cr_2O_7$ added-half the volume (ml) of N/2 $FeSo_4$ used.

3.18. ESTIMATION OF ORGANIC MATTER

% Organic Matter = % Organic Carbon×1.724.

3.19. ESTIMATION OF AVAILABLE MACRO-NUTRIENTS

3.19.1. Available Nitrogen

Available nitrogen from soil was estimated using an alkaline potassium permanganate solution as per the method of Association of Official Agricultural Chemists (AOAC), 1960.

Reagents

1. 0.32% Potassium permanganate solution ($KMnO_4$).
2. 2.5% Sodium hydroxide solution (NaOH).
3. 0.02N Sodium hydroxide (NaOH).
4. 0.02N Sulphuric acid (H_2SO_4).
5. Methyl red indicator (0.5 in 100 ml alcohol).

Procedure

Transferred 20 g soil sample in 800 ml Kjeldahl flask and added 20 ml of pure water to it. To these contents, added 100 ml of 0.32% $KMnO_4$ and 100 ml 2.5% NaOH in it. A few glass beads and 2-3 ml of paraffin liquid were also added to avoid contact with the upper part of the neck of the flask. Took 20 ml of 0.02N H_2SO_4 in a conical flask and added 2-3 drops of methyl red indicator and dipped the end of the delivery tube into it. Heated the contents until 100 ml of distillate was collected. Removed conical flask containing distillate and titrated it against 0.02N H_2SO_4 taken in the burette until the pink colour disappeared.

Calculations

Weight of the soil sample taken = 20 g.

Volume of 0.02N H_2SO_4 taken = 20 ml.

Volume of 0.02N NaOH used = X ml.

Volume of 0.02N H_2SO_4 used for absorbing NH_3 = (20-X) ml.

Available nitrogen = (20-X)×20 kg/ha.

3.19.2. Available Phosphorus

Method of Olson *et al.,* 1954 was followed for the estimation of available phosphorus.

Reagents

1. N/2 $NaHCO_3$-Dissolved 42 g of $NaHCO_3$ (AR) in one litre of water and adjusted the pH to 8.5 with 10N NaOH.
2. Ammonium molybdate solution – Dissolved 25 g of ammonium molybdate in 200 ml water, if necessary by warming. Diluted 275 ml of concentrated H_2SO_4 with 500 ml of water and cooled. Poured the molybdate solution into acid by stirring and made the final volume to 1 liter after cooling.
3. Stannous chloride – Dissolved 100 mg of stannous chloride (AR grade) in 5 ml of concentrated HCl by warming. Diluted to 10 ml with water in a test tube and added 1 cm layer of paraffin oil to prevent oxidation and wrapped a brown paper around the test tube.
4. *p*-Nitrophenol – 0.5% aqueous solution.
5. H_2SO_4 – 1+4, prepared by 1 volume of concentrated H_2SO_4 with 4 volumes of water.
6. Darco-G 60.
7. Standard – Dissolved 2.1965 g of KH_2PO_4 (AR grade) in water. Added 25 ml of (1+4) H_2SO_4 and made the volume 1 litre. The solution contains 500 ppm of phosphorus. Further dilutions were made from this stock solution.

Procedure

Took 5 g of soil sample in 250 ml flask and added 1-2 teaspoon of Darco-G 60. Added 100 ml of $NaHCO_3$ solution to it. Shook for 5 min. with hand and then on an electric shaker for half an hour and filtered it with Whatman no. 41 filter paper. To 20 ml of filtrate in a 50 ml volumetric flask, added 2 drops of *p*-nitrophenol indicator and neutralized it with diluted (1+4) H_2SO_4. Diluted to 40 ml with pure water and added 2 ml of ammonium molybdate reagent and made the final volume of 50 ml and transferred it into 100 ml conical flask. Developed the colour by adding 0.12 ml $SnCl_2$ and read the transmittance at 660 nm between 6-12 min. taking water as reference. Made a calibration curve from standard and used it to determine available phosphorus in the sample.

Calculation

Weight of soil sample taken = 5 g.

Volume of N/2 $NaHO_3$ used = 100 ml.

Volume of filtrate taken = 20 ml.

Final volume made for colour development = 50 ml.

Total dilution = 20×2.5 = 50 times.

Concentration of phosphorus read from standard curve = Y ppm.

Available phosphorus = Y×dilution = Y×50 (ppm).

= Y×50×2 kg/ha.

3.19.3. Available Potassium and Sodium

Available potassium and sodium were estimated, followed the method of Bower and Gschwend, 1952.

Reagents

1. Ammonium acetate solution – Dissolved 77.09 g of ammonium acetate in pure water and made the volume to 1 litre. Adjusted to pH 7.0 with ammonium hydroxide or acetic acid.
2. Working standards – Dissolved 1.9067 g of dry KCl (AR grade) and 2.5419 g NaCl (AR grade) in water and made the final volume to 1 litre, separately. The solutions contained 1000 ppm. Diluted this stock solution to produce a suitable range of concentrations between 0-100 ppm.

Procedure

Took 1g of soil sample in 100 ml conical flask and added 25 ml of neutral normal ammonium acetate solution. Shook the contents for 5 min. on an electric shaker and filtered through Whatman no. 40. The filtrate was put in the atomizer of the flame photometer and then readings of the samples were noted. The amount of potassium and sodium were calculated as given below

Calculations

Weight of soil taken = 1 g.

Volume of ammonium acetate added = 25 ml.

Dilution = 25 times.

Concentrated read from flame photometer = Y ppm.

Available K and Na = Y×25 (ppm).

3.19.4. Available Ca, Mg, Cl and HCO_3

For these, the methods given by Black, 1973 were followed.

3.20. PREPARATION OF SOIL EXTRACTS FOR DETERMINATION OF Ca, Mg, Cl AND HCO_3

For the preparation of aqueous soil extracts, 30 g of soil was taken in 500 ml conical flask and to this added 150 ml of pure water. Shook the contents for 1 hour on an electric shaker, allowed to stand for some time and filtered through Whatman no. 1. The filtrate was stored for estimations of Ca, Mg, Cl and HCO_3.

3.20.1. Available Calcium and Magnesium

Available Ca and Mg were estimated by following the Versenate or Disodium Dihydrogen Ethylenediamine Tetra Acetic Acid (EDTA) method.

Reagents

1. EDTA solution (0.01N) – Dissolved 2 g of EDTA in 900 ml water and made the final volume to 1 litre.
2. Ammonium chloride – Ammonium hydroxide buffer (pH 10) – 67.5 g ammonium chloride (AR grade) was dissolved in 570 ml of concentrated ammonium hydroxide and made volume to 1 litre and adjusted to pH 10.
3. Erichrome – Black T indicator – 0.5 g erichrome black T and 4.5 g of hydroxylamine hydrochloride (AR grade) dissolved in 100 ml of 95% ethyl alcohol.

Procedure

Took 5 ml extract and diluted to about 25 ml with pure water. Added one ml of ammonium chloride – ammonium hydroxide buffer and 3 to 4 drops of erichrome black T indicator. Titrated it with 0.01N EDTA solution until colour changed from wine red to blue or bluish green.

Calculations

$$\text{Milliequivalent (m.e.) of Ca+ Mg/litre} = \frac{\text{Vol. of EDTA used (ml)}}{\text{Vol. of aliquot used (ml)}} \times 10 = X$$

This is equivalent to 200 g soil.

So, m.e. of Ca + Mg/100 g soil = X/2

3.20.2. Available Calcium

Reagents

1. Sodium hydroxide 4N – Dissolved 160 g of NaOH (AR grade) in pure water and made volume to 1 litre.

2. Ammonium purpurate (mureoxide) indicator – Mixed 0.5 g of ammonium purpurate with 100 g of powdered potassium sulphate thoroughly.

Procedure

Took 5 ml extract in 100 ml conical flask and diluted to about 25 ml with pure water. Added 0.25 ml (5 drops) of 4N NaOH and approximately 50 mg of ammonium purpurate indicator and titrated it with 0.01N EDTA solution till the colour changed from orange red to purple.

Calculation

$$\text{Milliequivalent of Ca/ litre} = \frac{\text{Vol. of EDTA used (ml)}}{\text{Vol. of aliquot used (ml)}} \times 10 = Y = 200 \text{ g soil}$$

m.e. of Ca /100 g soil = Y/2

3.20.3. Available Magnesium

m.e. of Mg/100 g soil = X/2-Y/2

3.20.4. Available Chlorides

Chloride ions from the aqueous extracts were determined by the Chromate Titration Method.

Reagents

1. Silver nitrate (N/35.5) - Dissolved 4.785 g of silver nitrate in pure water and made the volume to 1 litre and stored it in amber coloured bottle to avoid oxidation.
2. Potassium chromate indicator –Dissolved 1 g of potassium chromate in 100 ml of pure water.

Procedure

Took 20 ml of soil extract in 100 ml conical flask and added 1 ml of potassium chromate solution, yellow colour developed. Titrated it against silver nitrate till the brick red precipitates appeared.

Calculations

$$\text{Milliequivalent of Cl/litre} = \frac{\text{Volume of } AgNO_3 \text{ used (ml)} \times \text{Normality of } AgNO_3}{\text{Volume of aliquot taken (ml)}} \times 1000$$

$$\text{m.e. of Cl/litre} = \frac{\text{Volume of } AgNO_3 \text{ used (ml)} \times \frac{1}{35.5} \times 100}{20} = 200 \text{ g soil}$$

$$\text{m.e. of Cl/litre} = \frac{\text{Volume of } AgNO_3 \text{ used (ml)} \times \frac{1}{35.5} \times 100}{20} \times \frac{1000}{2}$$

m.e. of Cl/100 g soil = volume of $AgNO_3$ used (ml) ×0.704

3.20.5. Available Bicarbonates

Bicarbonates were determined by titrating the solution with standard sulphuric acid.

Reagents

1. 0.1N H_2SO_4.
2. Methyl orange indicator - 0.1 g in 100 ml of 90% ethyl alcohol.

Procedure

Took 20 ml soil extract in 100 ml conical flask and added 2-3 drops of methyl orange indicator. Titrated it against 0.1N H_2SO_4 until colour changed from yellow to rose red.

Calculations

Miliequivalent of HCO_3/100g soil

$$= \frac{\text{Volume of } 0.1\text{N}\,H_2SO_4 \text{ used (ml)}}{10} \times \frac{\text{Volume of extract made (ml)}}{\text{Volume of extract used (ml)}} \times \frac{100}{\text{Wt. of soil}}$$

$$= \frac{\text{Volume of } 0.1\text{N}\,H_2SO_4 \text{ used (ml)}}{10} \times \frac{150}{20} \times \frac{100}{30}$$

m.e. HCO_3/100 g soil = Volume of 0.1N H_2SO_4 used (ml)×2.5

3.21. ESTIMATION OF AVAILABLE MICRONUTRIENTS (Fe, Mn, Zn and Cu)

The available forms of these micronutrients in soil were extracted with diethylene triamine penta acetic acid (DTPA). The content of these micronutrients in the extracted solution were analyzed on an atomic absorption spectrophotometer (AAS).

Reagents

DTPA extracting solution – Dissolved 1.967 g DTPA, 1.47 g of $CaCl_2H_2O$ and 13.3 ml of reagent grade triethanalamine (TEA), separately, in pure water and then mixed and diluted to 900 ml with pure water. Adjusted the pH to 7.4±0.05 with 1N HCl and made the volume to 1 litre.

Procedure

Took 20 g soil sample in a conical flask and added 40 ml DTPA solution to it and shook on an electric shaker for 2 hours, filtered it through Whatman filter paper no. 42, recorded the reading on AAS. Prepared standard curve for each micronutrient,

separately and calculated the concentration of the same (ppm) from the respective standard curve.

Calculations

Weight of soil taken = 20 g.

Volume of DTPA solution used = 40 ml.

Dilution = 2 times.

Reading of micronutrient on AAS = X.

Concentration of X on standard curve = Y ppm.

Micronutrient content in soil = Y×2 (in ppm).

3.22. DETERMINATION OF TOTAL PHENOLIC CONTENT FROM SOIL

For this, 1:5 (w/v) soil extracts (prepared above) were used, the amount of total phenolics was determined from 1 ml of these extracts using Swain and Hillis (1959) method as already given in detail.

3.22.1. PLANT ANALYSIS

3.22.1.1. Available Nitrogen

Total nitrogen content of plant tissues was determined by the Kjeldahl method.

Reagents

1. Concentrated sulphuric acid and 0.1N sulphuric acid.
2. Boric acid mixed indicator solution - Dissolved 20 g boric acid (AR Grade) in about 900 ml of hot water, cooled and added 20 ml of a mixed indicator solution (prepared by dissolving 0.1 g of boromocresol green and 0.07 g methyl red in 100 ml ethanol). Added 0.1N NaOH solution drop wise, until the colour was reddish purple and diluted to 1 litre with pure water.
3. Digestion accelerator mixture - Mixed 20 parts of anhydrous K_2SO_4 with 1 part of $CuSO_45H_2O$.
4. 40% Sodium hydroxide - Dissolved 40 g of NaOH (AR Grade) in 900 ml water cooled and made the volume 1 litre.

Procedure

1. Digestion of the Plant material – Weighed 0.5 g of finely ground, dried plant sample and dropped into 800 ml Kjeldahl flask. Added 20 g of a digestion accelerator mixture and 35 ml of concentrated H_2SO_4. Placed the flask in the digestion unit. Digested the content of the flask on a low heat to avoid frothing. After about 15-20 min., gradually raised the heat until the contents

become clear and pale green or blue coloured. Cooled the contents and made the volume 100 ml.

2. Distillation and Titration - Took 10 ml of liquid into 800 ml Kjeldahl flask and cooled about 300 ml of pure water and swirled the flask a little to mix and again cooled at room temperature. Took 25 ml of boric acid mixed indicator solution in a 250 ml conical flask and placed it under the ammonia receiving tube of the distillation assembly. Added a few glass beads and about 3-4 ml of paraffin liquid to the diluted and cooled sample. Added 100 ml of 40% NaOH solution slowly, along the sides of the distillation flask and attached the distillation unit. Continued distillation for about 30-40 min. After collecting about 100 ml distillate in a conical flask, removed the conical flask before switching off the heater. Titrated the distillate against 0.1N H_2SO_4 until a purple colour just starts appearing.

Calculations

Weight of the plant material taken = 0.5 g.

Normality of H_2SO_4 = 0.1.

Volume made after digestion = 100 ml.

Volume taken for distillation = 10 ml.

Volume of H_2SO_4 used in titration = X ml.

Milliequivalent of H_2SO_4 = 1.4 mg nitrogen = (0.0014g nitrogen).

m.e. of nitrogen/100 g of plant sample =

$$= X \times 0.1 \times 0.0014 \times \frac{100}{\text{Wt. of the plant material (g)}} \times \frac{\text{Volume taken for distillation (ml)}}{\text{Volume made after digestion (ml)}}$$

$= X \times 0.1 \times 0.0014 \times 100/0.5 \times 10/100$

m.e. of nitrogen/ 100 g of plant sample = $X \times 0.003$.

3.23. DIGESTION OF PLANT MATERIAL FOR ANALYSIS OF NUTRIENTS OTHER THAN NITROGEN

3.23.1. Di-acid Digestion

Reagents

1. Concentrated HNO_3 (AR Grade).
2. 60% $HClO_4$ (AR Grade).
3. 2N HCl (AR Grade).

Procedure

Took 2 g dried plant material in 800 ml Kjeldahl flask and added 20 ml of concentrated HNO_3 and 2-3 ml $HClO_4$. Put the flask on a hot plate in acid proof digestion chamber having fume exhaust system. Digestion was continued until the contents become colourless and only of the white dense fumes appeared. In case, the liquid turned brown added another 5 ml of the acid mixture for digestion. The acid contents become reduced to about 2-3 ml by continuing heating at the same temperature. Then, the flask was removed from the hot plate, cooled and added 10 ml of dilute colourless 2N HCl. Filtered the contents through Whatman no. 42 filter paper into 100 ml volumetric flask and after 3-4 washing with pure water, made the final volume the 100 ml.

Weight of the plant material taken = 2 g.

Volume made after digestion = 100 ml.

3.23.2. Available Phosphorus

Reagents

1. Vanadate- mollybdate reagent – Solution 'A' was prepared by dissolving 25 g of ammonium molybdate in 400 ml warm pure water and cooled. Solution 'B' was prepared by dissolving 1.25 g of ammonium metavanadate in 300 ml of boiling water, cooled and added 250 ml concentrated HNO_3 in it and cooled. Then added solution 'A' into solution 'B' and made the final volume of one litre.
2. Phosphate standard – Dissolved 0.2195 g of KH_2PO_4 to water and made the volume 1 litre. This solution contained 500 ppm phosphorus.

Procedure

Transferred 2 ml or suitable volume in a 50 ml volumetric flask and added 10 ml of vanadate molybdate solution and made the final volume with pure water and mixed thoroughly. The colour was developed fully in about 30 min. and then the intensity of yellow colour formed was read on a colorimeter at a wavelength 420 nm.

Standard curves were prepared by taking 0,1,2,3,4 and 5 ml of 50 ppm standard phosphorus in 50 ml volumetric flask and proceed in the same way as described above.

Calculations

Volume of aliquot taken for analysis = 2 ml.

Final volume made = 50 ml.

Transmittance (%) as read from the colorimeter = T.

P as read from the standard curve against T = Y ppm.

$$P = \frac{\text{Value read from st. curve (ppm)}}{\text{Wt. of the plant sample taken (g)}} \times \frac{100}{\text{Vol. of aliquot taken (ml)}} \times \frac{\text{Vol. made after digestion (ml)}}{10{,}000}$$

% P = Y/2×100/2×100/10,000

% P = Y/4

3.23.3. Available Potassium and Sodium

The amount of potassium and sodium in the acid digested plant was determined using flame photometer directly or after making a suitable dilution in the manner as described for K and Na in soil samples.

Calculations

$$\% \text{ K and Na} = \frac{\text{Value read from flame photometer} \times \text{Volume made after digestion}}{\text{Wt. of plant sample taken (g)}} \times \frac{100}{10{,}00{,}000}$$

$$= \frac{\text{Value read from flame photometer (ppm)}}{2} \times 100 \times \frac{100}{10{,}00{,}000}$$

$$\% \text{ K and Na} = \frac{\text{Value read from flame photometer (ppm)}}{200}$$

3.23.4. Available Calcium and Magnesium

Available calcium and magnesium in the acid digested plant sample was determined by versenate method exactly in the same manner as described for determination of available Ca and Mg in the soil extracts.

Calculations

Weight of the plant material = 2 g.

Volume of plant digests made = V (100 ml).

Volume of aliquot taken = V_1 ml.

Normality of EDTA used = 0.01.

Available Calcium + Magnesium

Volume of EDTA used in titration = V_2 ml

m.e. of (Ca + Mg)/100 g of plant sample =

$$V \times V_2 \times \frac{\text{Vol. of plant digest made (ml)}}{V_1} \times \frac{100}{\text{Weight of plant sample (g)}} \times X$$

Available Calcium

Volume of EDTA used in titration = V_3 ml.

m.e. of Ca/100 g plant sample =

$$V \times V_3 \times \frac{\text{Vol. of plant digest made (ml)}}{V_1} \times \frac{100}{\text{Weight of plant sample (g)}} \times Y$$

Available Magnesium

m.e. of Mg/100g plant sample = (X-Y) = X

3.23.5. Available Micronutrients (Fe, Zn, Mn and Cu)

Micronutrients were determined in acid digested plant material in ppm directly using AAS against known concentration of each standard.

3.24. ENERGY DISPERSIVE X-RAY SPECTROMETER SCANNING ELECTRON MICROSCOPY (SEM-EDX) OF RHIZOSPHERE SOIL

The elemental analysis of rhizosphere soil was performed at (USIF) University Sophisticated Instrument Facility, A.M.U., Aligarh. Following the collection of rhizosphere soil of *C. procera*, it was brought to the laboratory and dried in oven at 60ºC for four hours to remove moisture content. Dried samples were ground into fine powder using agate mortar. These samples (<1mm) were taken to the experimental section of USIF, placed on an Al stub, fixed with adhesive tape, coated with minimal amount of gold-palladium and analysed for element composition by SEM-EDX (Scanning Electron Microscope-Energy Dispersive Spectrometer) analysis at a minimum distance of 8 mm with 20KeV and 500X magnification. The microphotographs were recorded using the SEM JEOL (Model JSM-6510LV) with an accelerating voltage of 20keV at high vacuum (HV) mode and secondary electron image (SEI). The semi quantification elemental analysis to identify the weight percentage of major and minor elements present in the samples was done using the OXFORD INCA SEM-EDS. The relative intensities of the diffraction maxima were used for a semiquantitative estimation of the concentration of the mineral species present. This technique is being used in numerous applications for environmental science and technology.

3.25. SCANNING ELECTRON MICROSCOPY OF BIOLOGICAL SAMPLES

The varying magnifications of the adaxial and abaxial surfaces of *C. procera* were performed by using the JEOL (JSM-6510LV) SEM (Plate 5A) operated at 10keV acceleration voltage. The *C. procera* was harvested during its flowering stage from a natural population around the Aligarh Muslim University campus, Aligarh. Plant identification was done by an expert (Plant taxonomist) and a voucher no. 541 was deposited in the herbarium of Department of Botany, A.M.U., Aligarh. The methodology adopted by (Vaishali *et al.*, 2008; Gulzar *et al.*, 2015a) was used to examine the foliar ultramorphology following the general procedures. Freshly cut leaf samples were rinsed in distilled water and sectioned into about 4-6 mm segments before fixing in 0.05 M sodium cacodylate and rinsed again in 0.05 M cacodylate buffer (pH 7.5). Dehydration of the samples was performed by passing through a graded series of ethanol (20-100%) three times at 20 min. per rinse. This was followed by critical point drying with liquid carbon-dioxide in Hitachi HCP-2 Critical Point Dryer (Plate 5B). For mounting of each dried sample, aluminum specimen stubs with double-sided carbon coated adhesive discs and sputter coated with gold-palladium (Eiko IB-3 Ion Coater) was preferred. The JEOL (JSM-6510LV) SEM were operated at 10keV for examining adaxial and abaxial surfaces of the leaf specimen at varying magnifications. All the representative features examined were captured digitally using Microsoft Image Software for windows. The foliar ultrastructural morphology of leaf aqueous extract treated samples of *C. sophere* was also performed in the same manner.

3.26. CYTOGENETIC ASSAY DETERMINATION

Healthy and equal-sized bulbs of *Allium cepa* L. were used for cytogenetic experiments. Effect of leaf aqueous extract on mitotic activity was studied in onion root tips using the squash technique (Batish *et al*., 2006c; Teerarak *et al*., 2010). For four days, onion bulbs were grown in water to raise roots. The newly emerged roots were treated with 0.5%,1%,2% and 4% of leaf aqueous extract on 5^{th} day for 24 hours and distilled water was used as control. Next day, at the end of exposure period, the roots tips were cut and fixed in glacial acetic acid-ethyl alcohol (1:3 v/v) for another 24 hours after the removal of residue extract or water. Thereafter, rinsing with distilled water three times followed by hydrolysis with 1N hydrochloric acid for 1 min. at room temperature were performed. Staining with Schiff's reagent for 30 min.

were followed by macerating the two root tips in one drop of 40% glacial acetic acid on a slide. The slides were covered with a cover slip and sealed with clear nail polish. Five replicates were maintained per treatment and the experiment was repeated. Mitotic stages were observed under a bright field microscope (Olymous, model CH20i, New Delhi, India). The mitotic index was calculated by the formula

$$\textbf{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\textbf{Mitotic abnormality} = \frac{\text{Total no. of abnormal cells}}{\text{Total no. of cells scored}} \times 100$$

3.27. STATISTICAL ANALYSIS

Each experiment was performed in randomized block design and repeated. Sampling of the material from different experiments involving root and shoot length, seedling dry weight, nutrient analysis were made randomly both in control and treated samples, respectively. In each case, at least five replicates were maintained.

The data from the bio-efficacy studies, growth experiments and nutrient samples were analyzed by ANOVA and mean were separated at $P< 0.05$ and $P< 0.01$ by Duncan Multiple Range Test (Duncan, 1955). In case where a comparison of treatment with control was made, two-sample student's t-test was applied and presented only when the difference was significant. Besides, the data were subjected to determination of correlation coefficients between treatment and their respective concentrations wherever necessary.

(i) Mean

The arithmetic mean is the sum of the number of values divided by the total number of observations.

(ii) Standard Error

It determines the statistical significance of values obtained and is a measure of variability or of dispersion, which is the positive square root of the mean of the square of the deviation of the individual observations from their arithmetic mean.

(iii) Least significant difference

LSD is a method for comparing treatment group means. If the difference between the averages of two treatments exceeds the estimated LSD at 5% or 1% level the difference between those treatments is said to be significant at 5% or 1% level.

Chapter-4

Section I
Rhizosphere Soil

4. OBJECTIVE

To study various ecological features of *Calotropis procera* (Ait.) R. Br., its allelopathic impact and physico-chemical characteristics of rhizosphere soil.

4.1. OBSERVATION PARAMETERS

The following observations were made:

1. Physical measurements and biomass evaluation of different parts of *C. procera* at vegetative and flowering stage on over 100 plants.
2. Physico-chemical characteristics like composition, pH, electrical conductivity and element status of the soil collected from thick stands of *C. procera* during vegetative and flowering stage.
3. The impact of rhizosphere soil of the weed on a cumulative growth (in terms of seedling growth and dry biomass per seedling) of few crops, i.e. *Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., *Spinacea oleracea* L., *Pisum sativum* L. and weed plants, i.e. *Cassia sophera* L., *Cassia tora* L., *Chenopodium album* L. and *Cannabis sativa* L.
4. Rhizochemical detection by energy dispersive X-ray scanning electron microscope (EDX-SEM) analysis.
5. Identification of phenolic acids from rhizosphere soil.

4.2. MATERIALS AND METHODS

4.2.1. Biotic features and their measurement

At vegetative and flowering stage, the plants of *C. procera* growing locally were uprooted for measurement of features like average height of the ground part, primary root, secondary roots, average number of leaves per plant, etc. With the help of measuring tape, these parameters were measured. Oven drying method was followed for determination of the dry weight of the samples. The numbers of secondary roots, inflorescence, seeds, etc. were counted directly. Using venier caliper, determination of basal area of the stem (small), lengths and widths of the seeds was performed. For measurement of any of the feature, 100 plant samples were used.

4.2.2. Collection of soil and physico-chemical characteristic analysis

For the collection of soil, two sites were selected, one invaded by *C. procera* and other free from it. The former served as rhizosphere soil or *C. procera* infested soil and latter as control soil. Removing the vegetation followed the collection of soil from at least 10 locations in the study area.

After drying, grinding and sieving (to remove pebbles and other impurities) these soils, their analysis was done for estimation of pH, electrical conductivity, organic carbon, organic matter, phenolics and amount of macro-(O, N, P, K, Na, Ca, Mg, Cl and Si) and micro-(Zn, Fe, Mn, Cu and Al) elements. The method/instruments used for various physico-chemical characteristics and nutrient analysis have discussed in detail under (Chaper 3, Section Material and Methods). Besides, following the respective standard methods (see Materials and Methods'), elemental analysis of the plant samples at vegetative and flowering stages were made. The rhizosphere analysis of elements was also performed by Energy Dispersive X-ray Scanning Electron Microscope Analysis (EDX-SEM).

4.3. GROWTH STUDIES

From Indian Agriculture Research Institute (IARI), New Delhi and National Research Centre for Weed Science (NRCWS) Adhartal, Jabalpur (M.P.), seeds of crop plants, i.e. *T. aestivum, B. oleracea* var. *botrytis, S. oleracea, P. sativum* and weed plants, i.e. *C. sophera, C. tora, C. album, C. sativa,* respectively were procured for studying growth. They were subjected to growth studies in pots filled with soil samples from *C. procera* inhabited area as well as a control. Five replicates were maintained, for each test plant and treatment. The whole set-up was maintained under greenhouse conditions. Seedlings were uprooted carefully, after one month, keeping the root system intact. Their root and shoot lengths were measured and biomass quantified after oven drying.

4.4. IDENTIFICATION OF PHENOLICS IN THE RHIZOSPHERE SOIL (Gulzar and Siddiqui, 2015).

Samples of the rhizosphere soil from the *C. procera* plants were mixed thoroughly and sieved (2 mm mesh) to remove root tissue. One hundred grams of this oven-dried soil (at 35^0C) was extracted with 300 ml methanol (agitation, 48 hours at 25^0C, centrifugation, 1200×g for 30 min.). Pure methanol, a polar solvent, was used to extract the free phenolic acids from the soil because of its high extraction efficiency for the hydrophilic compounds (Kong *et al*., 2006). Furthermore, the methanol has a protective role, because it can prevent phenolic compounds from being oxidized by enzymes such as phenoloxidases (Proestos *et al*., 2006). The extracts were concentrated under vacuum at 40^0C and the residues were dissolved in methanol (6 ml) and filtered through a 0.45 mm filters prior to injection of 2 ml into the HPLC

system (HP 1200). The HPLC was equipped with a reverse-phase Zorbax SB-C18 column (eclipse 100 mm×2.1 mm, 1.8 mm) with a diode array detector. The temperature of the column oven was set at 35^0C. For the analysis, a linear gradient elution was used, with the mobile phases of acetonitrile (solution A) and aqueous 1% acetic acid (solution B), as follows: 100% solvent B at 0 min., 85% solvent B at 12 min., 50% solvent B at 20 min., 0% solvent B at 22 min., 100% solvent B at 24 min., isocratic elution of 100% B, 24-30 min. The flow rate was 0.4 ml/min, with detection at 280 nm. Phenolic acids were identified by comparing their retention time with those of the standards (procured from Sigma, St. Louis and Lancaster, UK).

4.5. STATISTICAL ANALYSIS

ANOVA followed by Duncan's Multiple Range Test (DMRT) as per Duncan (1955) were used for analysis of data and 2 sample t-test, wherever applicable.

4.6. RESULTS

4.6.1. Variation in growth characteristics between vegetative and flowering stage

Between vegetative and flowering stages, plants of *C. procera* exhibited variation about many of its characteristics. Aerial coverage area in the vegetative stage was (0.020±0.01 m^2) and at flowering stage, it was (1.10±0.11 m^2) (Table 1.1). The rhizosphere area (indicating the spread of roots) was (61.26±2.73 cm^2) and it increased to (128.19±19.20 cm^2) at flowering stage (Table1.1). The percent change from vegetative to flowering stage was about (109.25%). However, at the vegetative stage after bolting under favourable conditions, the leaf number was counted to be (391.00±92.69). The number of leaves was found to be still more (nearly four times) at the flowering stage nearly (157%) (Table1.1). The number of branches increased from (14.88±1.70) (during vegetative stage) to (21.41±1.12) (during flowering stage), it increased about (43.88%). The height of the plants (from the soil surface till the tip) at vegetative stage was (23.37±2.46 cm), which increased to about (95.49±6.37 cm) at flowering stage exhibiting an increase of about (308.60%). The length of the primary root also increased from (50.13±1.86 cm) to (83.67±5.68 cm) during the one-month period of change from vegetative to flowering stage (Table1.1). Likewise, the average length of secondary roots and tertiary roots (19.42±1.08 cm) and (11.26±1.02 cm) measured during vegetative stage was found to increase at flowering stage (34.13±1.75 cm) and (20.11±1.74 cm) by about (75.74%) and (78.59%).

The average fresh weight of the aboveground one month plant at vegetative stage was (99.13±0.97 g). In a span of two months when plants changed from a vegetative stage to flowering stage, the fresh weight increased to (270.73±48.96 g) showing an increase of about (173.10%) (Table 1.1).

The stem at vegetative stage was less in biomass with an average value of (21.30±0.54 g/plant). When the plant matured, the biomass was measured (172.47±19.64 g) increased abruptly. The change in fresh weight of the stem was drastic. The fresh weight of the leaves in vegetative phase was (24.78±0.55 g) compared to (46.23±0.68 g) during flowering stage, approximately (86.56%) increase (Table 1.1). The increase in root fresh biomass from vegetative to flowering stage was also noticeable. During the vegetative stage, it was (12.27±0.59 g), which increased to (33.57±1.70 g) during flowering stage (Table 1.1).

The dry biomass of the above ground plant parts during vegetative stage was (12.49±0.52 g/plant) while at flowering stage it becomes (40.20±0.24 g/plant). The values of different parts of the plant, i.e. stem, leaves and root during vegetative stage were (8.49±0.48 g), (10.93±0.16 g) and (4.97±0.17g), respectively. The corresponding values of these plant parts during flowering stage were (11.66±0.47 g), (24.95±0.15 g) and (15.43±0.61 g), indicating approximately (37.33%), (128.27%) and (210.43%) increase, respectively (Table1.1). The flowers of C. *procera* were shallowly campanulate and bearing on an average (150.13±2.05) inflorescences/plant while the average number of flowers were (10.84±2.03) and average diameter of the flower was also measured 1.86±0.15 with fresh and dry biomass of (19.17±2.71 g) and (10.35±0.65 g), respectively (Table 1.1).

On an average, number of seeds/plant, (98±2.00) could be counted. Each seed measured about (5.66±0.55 mm) in length and (2.92± 0.90 mm) wide, with a weight (1.34± 0.54 g/100 seeds) (Table 1.1).

Table 1.1: Biotic features of *C. procera* collected from invasion site*

Features	Vegetative stage	Flowering stage
Growth Features		
Rhizosphere area (cm^2)	61.266±2.73	128.19±19.20
Basal area (cm^2)	18.93±5.94	28.92±4.44
Aerial spread (m^2)	0.020±0.01	1.10±0.11
Average number		
(a) Leaves/plant	391.00±92.69	1008.3±25.65
(b) Branches /plant	14.88±1.70	21.41±1.12
Average length (cm)		
(a) Above ground part	23.37±2.46	95.49±6.37
(b) Primary root	50.13±1.86	83.67±5.68
(c) Secondary root	19.42±1.08	34.13±1.75
(d) Tertiary root	11.26±1.02	20.11±1.74
Fresh biomass (g)/ plant		
(a) Above ground part	99.13±0.97	270.73±48.96
(b) Stem	21.30±0.54	172.47±19.64
(c) Leaves	24.78±0.55	46.23±0.68
(d) Roots	12.27±0.59	33.57±1.70
Dry biomass (g)/plant		
(a) Above ground part	12.49±0.52	40.20±0.24
(b) Stem	8.49±0.48	11.66±0.47
(c) Leaves	10.93±0.16	24.95±0.15
(d) Root	4.97±0.17	15.43±0.61
Inflorescence		
(a) Number of inflorescences / Plant	.	150.13±2.05
(b) Number of flowers/ inflorescence		10.84±2.03
(c) flowers diameter(mm)		1.86±0.15
(d) Fresh biomass/plant(g)		19.17±2.71
(e) Dry biomass/plant(g)		10.35±0.65
Seeds		
(a) Number/plant		98.00±2.00
(b) Length (mm)		5.66±0.55
(c) Width (mm)		2.926±0.90
(d) Weight of 100 seeds(mg)		1.34±0.54

***** The data between the pre-and post-flowering stage were significantly different applying 2 sample t-tests

± represent standard deviation.

4.7. ALLELOPATHIC EFFECT OF C. PROCERA INVADED SOIL ON GROWTH RESPONSE OF TEST SPECIES

4.7.1. Germination

Germination of seeds of each test plant (crops and weeds) in the soil, collected from *Calotropis* invaded field as well as the control were noticed. Since, there has been no change in germination. Data have not been presented,

4.7.2. Root length

Generally, the root length of test plants emerging from the seeds sown in the rhizosphere soil of C. *procera* was shorter than those of controls. In control, the root length of *T. aestivum* was found to be (29.86±0.45 cm). Their root length was measured to be (26.79±0.61 cm) reduced by (15.06%) when grown in soil collected from *C. procera* invaded area as compared to control (Fig. 1.1a). This reduction was statistically significant. Root length in soil collected from *C. procera* invaded field was (20.19±0.82 cm) compared to (23.07±1.29 cm) in control, reduced by (23.69%) (Fig. 1.1a) in case of *P. sativum*. While, the reduction in root length was also noticed to be (13.96±0.38 cm) and (10.31±0.66 cm) in rhizosphere soil in relation to control soil where values were (18.00±0.96 cm) and (12.62±0.56 cm), i.e. reduced by 12.86% and 29.42% in the case of *S. oleracea* and *B. oleracea* var. *botrytis*. Similarly in weeds (*C. tora, C. sophera, C.album* and *C. sativa),* the root length was reduced by (35.67%), (26.92%), (40.78%) and (27.21%) (Fig. 1.1a). In *C. procera* invaded soil, the observed values of their root length are (5.88±0.51 cm), (7.34±0.62 cm), (2.47±0.74 cm) and (6.10±0.56 cm) as compared in control soil (7.96±0.15 cm), (9.36±0.96 cm), (5.34±0.86 cm) and (8.77±0.56 cm).

4.7.3. Shoot length

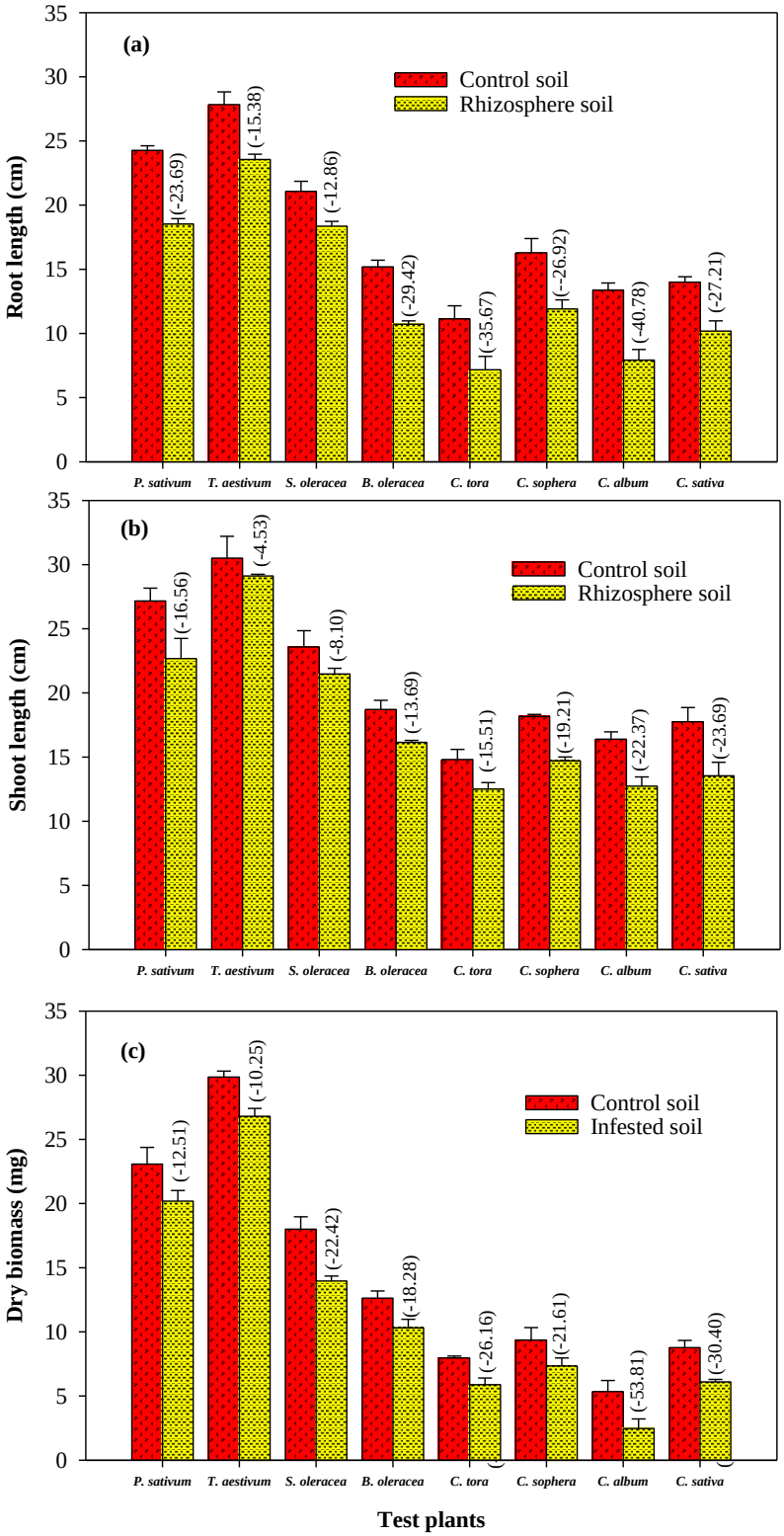
In *C. procera* infested soil, the shoot length of each test plant grown was less than the respective values of those grown in soil collected from *C. procera* free area or control. In case of *T. aestivum,* when grown in control, the shoot length of seedlings was measured to be (30.50±1.00 cm) (Fig. 1.1b). Compared to it, when grown in the soil collected from *C. procera* infested area, the shoot length was (29.12±0.12 cm) (Fig. 1.1b) reduced by (4.53%). In *C. procera* invaded soil, shoot length was reduced by (16.56%) as it was (22.67±1.56 cm) in comparison to (27.17±1.00 cm) in the control soil in case of *P. sativum* (Fig. 1.1b). When grown in invaded field soil while in the case of *S. oleracea* and *B. oleracea* var. *botrytis,* the

shoot length was reduced by 8.10% (21.47±0.44 cm) and 13.69% (16.14±0.15 cm), respectively in comparison to control soil (Fig. 1.1b). Among weed plants, maximum reduction (22.69%) was observed in *C. sativa,* i.e. (13.54±1.04 cm) compared to (17.75±1.11 cm) in control. It was followed by (22.37%), (19.21%) and (15.51%) reduction in *C. album, C. sophera* and *C. tora* compared with the values of (16.39±0.16 cm), (18.16±0.79 cm) and (14.81±0.71 cm), respectively in control soil. This reduction was statistically significant.

4.7.4. Dry Biomass

As compared to control, the dry biomass of seedling grown in *C. procera* invaded soil was less. Among crop plants, maximum reduction was observed was about (22.42%) in *S. oleracea* with the values in invaded soil (13.96±0.38 mg) compared to (18.00±0.96 mg) in control, but in *B. oleracea* var. *botrytis*, the reduction was (18.28%), in *P. sativum* (12.51%) while in *T. aestivum* (10.25%) with noticed values of (10.31±0.66 mg), (20.19±0.82 mg) and (26.79±0.61 mg), respectively. In the weed test plants maximum reduction was observed in *C. album* about (53.81%), followed by about (30.40%) in *C. sativa,* (26.16%) and (21.61%) in *C. tora* and *C. sophera*, respectively (Fig. 1.1c). In all cases, a significant reduction in plant dry weight was noticed.

Fig. 1.1: Allelopathic effect of rhizosphere soil of *C. procera* on (a) radicle length (b) plumule length and (c) dry biomass of recipient species (crops and weeds)*.



*represents the significant variation between the values in rhizosphere soil and in control soil applying 2 sample t- test.
Value in parenthesis represents % reduction of control.

4.8. ELEMENTAL COMPOSITION

A significant difference in the amount of the various elements was observed in *C. procera* at both vegetative and flowering stages. However, the variation was statistically significant at 5% level except in the case of Na, Cu and Cr (Table 1.2). The amount per unit dry weights at flowering stage was relatively more than that of vegetative stage in the case of all elements. Further, the amount was 0.034 to 0.084 of Na per unit dry weight from vegetative to flowering stage, i.e. an increase of (147.05%), among all elements (Table 1.2). In case of N, Mn, K, Ca, Mg, Pb and Zn, significant percent decrease in their amount (i.e. about 27.78%, 40.10%, 23.60%, 30.10%, 17.61%, 24.19%, 58.59% and 33.70%), respectively at vegetative stage compared to flowering stage occurred. There was a very less change in the amount (9.99%, 3.27% and 7.89%) during vegetative and flowering stage, respectively in the case of Fe, P and Cd.

Table 1.2: Elemental analysis of *C. procera* at vegetative and flowering stages.

Elements (units)	Vegetative stage	Flowering stage
N (%)	0.39	0.54
P (%)	0.59	0.61
K (%)	5.73	7.5
Na (%)	0.034^{ns}	0.084^{ns}
Ca (%)	40.17	57.47
Mg (%)	18.94	22.99
Zn (ppm)	2.38	3.59
Fe (ppm)	19.00	21.11
Mn (ppm)	1.15	1.92
Cu (ppm)	0.17^{ns}	0.22^{ns}
Cd (ppm)	0.70	0.76
Pb (ppm)	0.47	0.62
Cr (ppm)	0.13^{ns}	0.20^{ns}
LSD at 5%	**0.59**	**0.41**

ns represent insignificant different at pre-flowering and post-flowering stage applying 2 sample t-test
± represent standard deviation.

4.9. SOIL CHARACTERISTICS

Apart from the amount of macro and micro-nutrients, the soil collected from *C. procera* invaded area were analyzed for some physico-chemical characteristics. In general, the control as well as *C. procera* invaded area, the soils were slightly alkaline and a little statistically significant difference was observed among these soils (Table 1.3). However, the electrical conductivity was found maximum in *C. procera* invaded site at flowering stage followed in sequence by vegetative stage and control. The differences among these three were also statistically significant. The soils were also analyzed for phenolic content. In *C. procera* invaded site, the maximum amount of phenolics was found at vegetative stage followed by flowering stage and least in control. The differences were also statistically significant and with regard to organic carbon and organic matter, both were found maximum at vegetative stage followed by flowering stage and control (Table 1.3). Thus, in *C. procera* invaded site at vegetative stage, the amount of organic carbon was maximum followed by flowering stage and control.

The determination of macro and micro-nutrients amount were also assessed in soils under observation (i.e. vegetative, flowering and control). Generally, at flowering stage in *C. procera* invaded site, the maximum amount of elements was calculated followed by vegetative stage and control. However, in the content of Mg, Zn, Fe, Mn and Cu, the maximum amount of respective element or nutrient was found in soil at flowering stage, followed by vegetative stage and control. The differences were found to be statistically significant, in the amount of almost all macro and micro-nutrients among the control, vegetative and flowering stage (Table 1.3).

Table 1.3: General Characteristics of soil collected from *C. procera* infested area.

Soil characters	Control	Vegetative stage	Flowering stage	LSD at 5%
pH	7.60±0.03[a]	7.36±0.06[b]	7.21±0.10[c]	**0.29**
Conductivity (µS)	157.17±9.53[a]	226.30±52.11[a]	229.50±46.42[a]	**170.6**
Phenolic content (mg/100g soil)	0.85±0.13[b]	2.11±0.29[a]	1.66±0.33[a]	**1.13**
OC (%)	1.10±0.02[c]	1.95±0.030[a]	1.74±0.037[b]	**0.12**
OM (%)	1.43±0.03[c]	3.21±0.02[a]	2.91±0.02[b]	**0.11**
N (kg/ha)	182.33±2.51[c]	210.00±2.00[b]	218.00±2.64[a]	**10.07**
P (kg/ha)	167.00±2.46[b]	94.33±2.51[c]	188.33±1.52[a]	**9.25**
K (ppm)	110.33±1.52[c]	148.67±3.51[b]	157.00±2.00[a]	**10.43**
Na (ppm)	42.66±2.67[b]	62.40±0.30[a]	64.38±0.24[a]	**6.52**
Ca (g/100g)	3.52±0.20[c]	7.45±.0.25[a]	5.31±0.02[b]	**0.79**
Mg (g/100g)	2.56±0.05[c]	4.71±0.11[b]	6.29±0.17[a]	**0.52**
Cl (g/100g)	4.52±0.21[a]	6.45±0.29[a]	4.57±2.01[a]	**4.96**
HCO_3 (g/100g)	14.70±0.03[c]	31.71±0.28[a]	22.62±0.18[b]	**0.82**
Zn (ppm)	2.68±0.29[c]	6.62±0.38[b]	7.53±0.44[a]	**1.57**
Fe (ppm)	6.49±0.44[c]	8.49±0.44[b]	11.41±0.33[a]	**1.73**
Mn (ppm)	11.21±0.10[c]	11.73±0.26[b]	13.56±0.19[a]	**0.83**
Cu (ppm)	0.22±0.10[b]	0.72±0.032[a]	0.78±0.010[a]	**0.26**

Similar superscript symbols along a row represent significant difference at P<0.05 applying DMRT
± represent standard deviation.

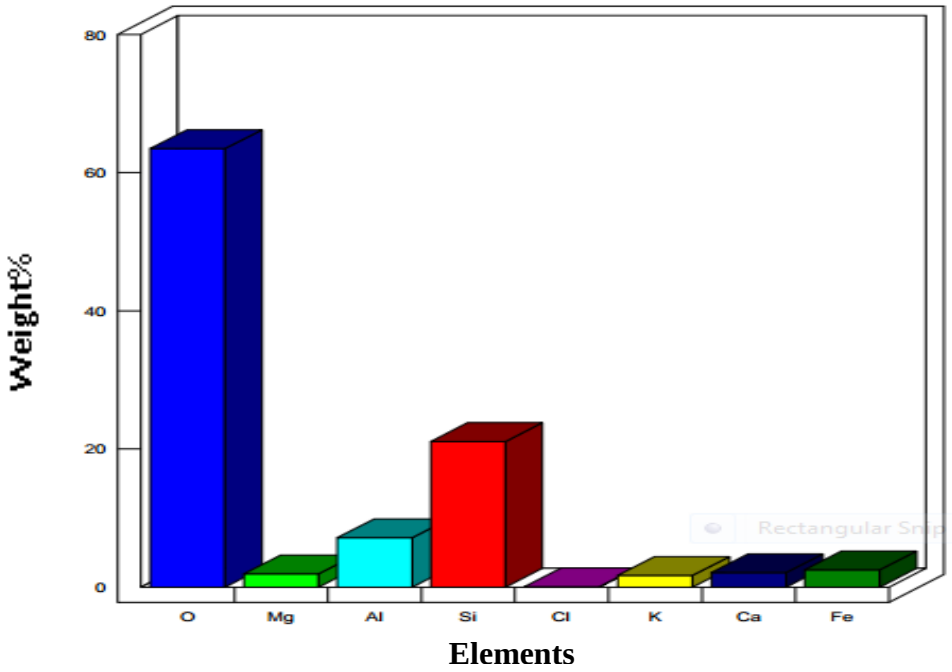
4.10. EDX-SEM ANALYSIS OF RHIZOSPHERE SOIL

Apart from analyzing the rhizosphere soil by standard methods, the SEM analysis also detected both macro and micronutrients in the rhizosphere soil of *C. procera* (Plate 1.1). The elements that were detected include O, Mg, Al, Si, K, Ca, Fe, Zn, N, Mn, Cu, Cl and P. The alkaline earth elements like O, Si, Al and Mg shows its highest presence in terms of atomic and weight percentage shown in Table 1.4 and Fig. 1.2. Among micro-nutrients (Cl, Cu, Fe, Mn, Zn), only two elements Fe and Cl were detected with their respective percentages. The typical microphotograph of detected elements of rhizosphere soil is presented in Plate 1.2.1.

Table 1.4: Rhizochemicals with their weight percentage and atomic percentage analysed by EDX- SEM analysis.

Element	Weight %	Atomic %
O	63.48	76.23
Mg	1.91	1.51
Al	7.15	5.09
Si	2.08	14.42
Cl	0.06	0.03
K	1.70	0.84
Ca	2.10	1.01
Fe	0.86	2.51
Totals	100%	

Fig 1.2: Quantitative determination of rhizoelements by EDX-SEM analysis.



4.11. IDENTIFICATION OF PHENOLIC ACIDS

Upon HPLC analysis of the rhizosphere soil, four phenolic acids were identified. These include ferulic acid, vanillic acid, *p*-coumaric acid and benzoic acid with retension times of (1.56, 2.00, 3.72 and 4.66 min.), respectively (Table 1.5). None of these, however, was present in the control soil.

Table 1.5: HPLC analyses of the phenolic acids in the rhizosphere soil of *C. procera.*

Phenolic acid	Retention time (min.)	Rhizosphere soil
Ferulic acid	1.56	18.12
Vanillic acid	2.00	14.52
p-coumaric acid	3.72	11.21
Benzoic acid	4.66	8.75

4.12. DISCUSSION

The comparison of features between vegetative and flowering stages indicates the extensive and profuse growth of the weed. The weed covers an area at both the ground level as well as the aerial level that increased appreciably from vegetative to flowering stage has been depicted from the basal area of the stem. From the present study, it can be observed that from vegetative to the flowering stage, number of leaves, height of the above ground parts, length of primary and secondary roots and fresh and dry biomass quickly increases. Maximum growth has been seen in both above and below ground parts of the plant. The weed grows very fast also suggested from fresh and dry weight of all these parameters. In Aligarh and its surroundings, *C. procera* is a very common weed and can be seen almost throughout the year. Its common occurrence and successful invasion is due to the following features such as fast growth and dissemination, high seed production dispersed efficiently by the wind, non-specialized pollution system and high tolerance to poor soils (Oliveira *et al*., 2009).

The rhizosphere soil with its allelopathic impact, indicates its retardatory effect on growth, the potential of which varied from species to species. The growth of test species when grown in *C. procera* infested soil was significantly affected compared to control as clearly depicted from the experiments. Both test plants (crops/weed) growth and biomass accumulation were significantly reduced in *C. procera* invaded soil. The studies indicate that some inhibitors are present in the

rhizosphere soil of *C. procera* that adversely affects the early growth of test plants compared to control. Generally, a greater retardatory effect was seen in *C. album* as compared to other species. Based on root length of the test plants, the decreasing order of sensitivity appeared to be *T. aestivum>P. sativum>S. oleracea>B. oleracea >C. sophera>C. tora>C. sativa>C. album.* On the basis of shoot length, the decreasing order of sensitivity in the test plants was shown *T. aestivum>P. sativum>S. oleracea>B. oleracea>C. sophera>C. album>C. sativa>C. tora.* In the case of dry weight, the decreasing order of sensitivity in the test plants *T. aestivum>P. sativum>S. oleracea>B. oleracea>C. sophera >C. sativa>C. tora>C. album.* Various recent studies (Batish *et al.,* 2006a, 2007a; Sisodia and Siddiqui, 2009; Raoof and Siddiqui, 2012a; Fragasso *et al*., 2012; Iannucci *et al*., 2013; Safdar *et al.,* 2014; Gulzar *et al*., 2011; Gulzar *et al.,* 2014c; Gulzar and Siddiqui, 2015; Li *et al.,* 2016) have evaluated the phytotoxic activity of rhizosphere soil that supports our finding.

The studies indicate that some inhibitors are present in the rhizosphere soil of *C. procera* that adversely affects the early growth of test plants compared to control. The presence of phenolics might be adversely affecting the growth of other plants (Sarkar and Chakraborty, 2010) grown in soil inhabited by *C. procera.* Likewise, the amount of all the nutrients (whether macro-or micro-or ions) was more in *C. procera* field soil compared to control soil and hence they are not responsible for growth retardatory effects of test plants. These results indicated the definite role of allelopathy of rhizosphere soil in retarding weed and crop growth as per study of Batish *et al*. (2007a). On the other hand, the phenolics were found in appreciable amount in rhizosphere soil from *Calotropis* invaded area compared to control in our study and several studies has indicated that these phenolics are responsible for the growth retardatory effect on other plants, including crops, thus causing appreciable injury in the growing plants (Rice, 1984, 1995; Qasem and Foy, 2001; Weston and Duke, 2003; Batish *et al*., 2007b).

In ecological terms, the rooting zone and rhizosphere is a very competitive environment where the roots of neighbouring species and microorganisms compete for space, water, nutrients and gases (Weston *et al.,* 2012). Roots also perform several more specialized roles in the rhizosphere, which rely on the synthesis and exudation of metabolites in addition to providing mechanical support, water and nutrients (Brigham *et al*., 1999; Bertin *et al*., 2003; Walker *et al*., 2003a; Weston *et al*.,

2012). Root secretions comprise the majority of low molecular mass constituents such as amino acids, organic acids, sugars, phenolics and other secondary metabolites (Bertin *et al*., 2003). As a result, exudates can repel herbivores and microbes, stimulate symbiotic relationships, alter soil properties and inhibit the growth of competing species (Nardi *et al*., 2000; Watt and Weston, 2009; Mathesius and Watt, 2011; Weston *et al*., 2012). Root produced allelochemicals are generally associated with the reduction in neighbouring plant growth and resistance to or suppression of plant pathogens, soil microbes and other herbivores (Weston *et al*., 2012). The presence of phenolics in the rhizosphere soil of *Calotropis* invaded fields indicates that these might have been released from the plants through any of the mode is in line with the study of Batish *et al*. (2007a).

Not much change in soil pH was observed between C. *procera* invaded site and the control soil. The pH was slightly alkaline or even near neutral. The electrical conductivity, however, was more in the soil collected from *C. procera* infested soil. The percent organic carbon and organic matter found to be maximum in soil supporting *C. procera* plants at vegetative stage followed by soil at flowering stage and control. The reason for this could be that foliage at vegetative stage was quite expanded and thus, its death and decay adds more organic matter and organic carbon and also the number of plants was more at this stage and when the plant reaches the flowering stage, its foliage gets comparatively smaller. It has largely been over taken by its inflorescence. The amount of N, P and K were measured to be substantially higher compared to that of control and it was even more during the flowering stage. Likewise, the amount of Na and Mg followed the same trend. Although the amount was more in case of chlorides, bicarbonates and calcium in contrast to control soil at vegetative and flowering stage, yet decrease was observed in soil with plants at vegetative stage compared to those at flowering stage. In case of micronutrients like Fe, Zn, Mn and Cu, a similar trend was observed as in case of N, P and K. Thus, it becomes clear from the study that soil supporting *C. procera* invaded plants either at vegetative and flowering stage is not deficient in any of nutrients rather the status of both macro and micronutrients are better in comparison to control favoring the better growth of *C. procera.* Besides, macro and micronutrients, the amount of total phenolics was also estimated in the soils. It was found to be maximum in soil at vegetative stage followed by that in soil with flowering stage and least or negligible in

control soil. The phytotoxic effect induced by rhizosphere soil of *C. procera* indicates that the allelopathic effect of the weed could be due to the presence of phenolics (Fragasso *et al.,* 2012; Iannucci *et al.,* 2013; Liu *et al.,* 2013; Liu *et al.,* 2014).

The plants of *C. procera* also possess an appreciable amount of N, P, K, Na, Ca, Mg and micro-nutrients like Zn, Fe, Mn and Cu. There also occurs the variation in the amount of micro and macronutrients at vegetative and flowering stage. Generally, the amount was more in plants at flowering stage compared to the vegetative stage. The detection of micronutrients (Fe, Mn, Zn and Cu) and macronutrients (Ca, K and Mg) in *C. procera* by (Naz and Bano, 2013) is in line with our findings. Likewise, the analysis of essential elements Al, As, Cu, Ca, Cr, Cd, Fe, K, Mn, Na, Pb and Zn in the variable range from the *C. procera* has been reported by Khanzada *et al.* (2008).

How these inhibitors or allelochemicals are released or accumulated in the soil, only speculations can be made. Rice (1984, 1995) pointed that leachation aided by natural agencies of water, decomposition, root exudation and volatilization are four possible modes of release of allelochemicals. Allelochemicals may be released through any of these or through all of these modes. Besides, retention, transformation and transport of allelopathic chemicals in soil and physicochemical and biological components of the soil can influence the fate of allelopathic chemicals and thus of allelopathy, in soil (Inderjit, 2001; Inderjit *et al.,* 2010; Iannucci *et al.,* 2013). Leachation and microbial decomposition in the present case may have caused the release of allelochemicals as the study was conducted during the rainy season where not only the leachation is quick but the rate of decomposition of fallen plant parts is also high. Further, because of quick multiplication and rapid growth, the allelochemicals are continuously being added to rhizosphere soil.

Upon HPLC analysis of rhizosphere soil, four phenolic acids were identified. (Gulzar *et al.,* 2015b; Ren *et al*., 2015; Liu *et al*., 2016). As reported by Turk and Tawaha (2003), phenolic acids are among the main category of allelochemicals in nature. These phenolic compounds can inhibit root elongation and cell division in plants and can cause changes to the cell ultrastructure, thus interfering with the normal growth and development of the whole plant (Liu *et al*., 2014). Based on the results, the study concludes that *C. procera* interferes with the growth of test species by releasing water-soluble phenolics and allelopathy is operative in the

community dominated by *C. procera* and may even provide an advantage to the weed.

Section JJ
Aqueous Extract

5. OBJECTIVE

To study the allelotoxicity of different parts of *C. procera* (Ait.) R. Br. collected at flowering stage on weed and crop plants.

5.1. OBSERVATION PARAMETERS

1. Treatment in response to different concentrations (0.5%,1%,2% and 4%) of extracts prepared from the roots, stem and leaves collected at flowering stage on growth in terms of seedling root length, shoot length and dry weight of the crop plants (*Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., *Spinacea oleracea* L., *Pisum sativum* L.) and weed plants (*Cassia sophera* L., *Cassia tora* L., *Chenopodium album* L. and *Cannabis sativa* L.) was studied.
2. Determination of pH, osmotic potential and total phenolic contents of extracts.
3. Foliar micro-morphological epidermal changes induced in *C. sophera* by leaf aqueous extract as revealed by scanning electron microscopy.

5.2. METHODOLOGY

5.2.1. Preparation of aqueous extract and and germination bioassay (Gulzar *et al*., 2015a)

At flowering stage, *C. procera* leaves, stem and roots were collected randomly from the campus of Aligarh Muslim University, Aligarh (27°, 29° to 28°, 100° NL and 77°, 29° to 78°, 38° EL). Plant identification was done by an expert (Plant taxonomist) and a voucher no. 541 was deposited in the herbarium of Department of Botany, A.M.U., Aligarh. They were brought to the laboratory, washed to remove the dirt and shade dried. Further, the dried leaves were ground to fine powder with the help of a grinder (Singh *et al*., 2002). The preparation of aqueous extract of plant parts and their phytotoxic effect through impact on germination and growth of the target species have been considered as a classic procedure in the field of allelopathy (Inderjit and Dakshini, 1995; Blum, 2011; Borghetti *et al*., 2013). Further, in allelopathic interference, the use of water has been recommended because it reflects more closely what would happen under natural conditions (Borghetti *et al*., 2013). A weighed amount of each plant material (4 g) was immersed in 100 ml of pure water for 16 hours at 24°C to obtain a stock solution (4%). The extract was filtered through a double layer of muslin cloth followed by a cheesecloth and finally through a Whatman no. 1 filter paper. The extract was diluted with distilled water to obtain concentrations of 0.5%,1% and 2% (w/v) and kept in a refrigerator at 4°C for 24

hours in darkness for the solubilization of active compounds (Borghetti *et al*., 2005; Pina *et al.,* 2009). The seeds of crops and weeds were procured from the Indian Agriculture Research Institute, New Delhi and National Research Centre for Weed Science, Jabalpur (MP) and were exposed to different concentrations of each part. For this, 15 seeds of test species were germinated in a 15-cm Petri dish lined with Whatman no. 1 filter paper and moistened with 15 ml of each extract or water (to serve as control). Petri dishes were placed in a growth chamber maintained at 25±2°C, 75±2% RH and 16/8-h light-dark photoperiod. Each treatment was maintained in a completely randomized block design with five replicates. Shoot length and root length were measured by using a meter scale after 15 days. The samples were dried in an oven at 72°C followed by dry biomass determination on a four-digit digital balance of Scientech, Model ZSA 120, Colorado (USA). For the confirmation of observations, the whole experiment was repeated.

5.2.2. SCANNING ELECTRON MICROSCOPY (Gulzar *et al.*, 2015a)

Scanning electron microscopy (SEM) was performed on 15 day old leaves of *C. Sophera* seedlings with a view to see alterations, if any, on the leaf surface upon exposure to the leaf aqueous extract. The methodology adopted by (Vaishali *et al.*, 2008) was used to examine the foliar ultramorphology following the general procedures. Leaf samples of *Cassia* seedlings were rinsed in distilled water and fixed into 0.05M sodium cacodylate and rinsed again in 0.05M cacodylate buffer (pH 7.5). Dehydration of the samples was performed by passing through a graded series of ethanol (30%,50%,70% and 80%) three times at 5 min. per rinse. This was followed by critical point drying with liquid carbon dioxide in Hitachi HCP-2 critical point dryer. For mounting of each dried sample, aluminum specimen stubs with double-sided carbon coated adhesive discs and sputter coated with gold palladium (Eiko IB-3 ion coater) were preferred. The JEOL (JSM-6510LV) SEM was operated at 10-15kV for examining at varying magnifications. All the representative features examined were captured digitally using Microsoft Image Software for windows.

5.3. STATISTICAL ANALYSIS

All the experiments were laid out in a completely randomized block design. Data of mean values were analyzed by ANOVA followed by DMRT. The data of root length, shoot length, dry biomass, pH, osmotic potential and total phenolic content were expressed with respect to control and analyzed by DMRT at $P<0.05$. The values

of correlation coefficients between concentration and respective parameters were also calculated.

5.4. RESULTS

5.4.1. Aqueous extract prepared from leaves of *C. procera* and its impact

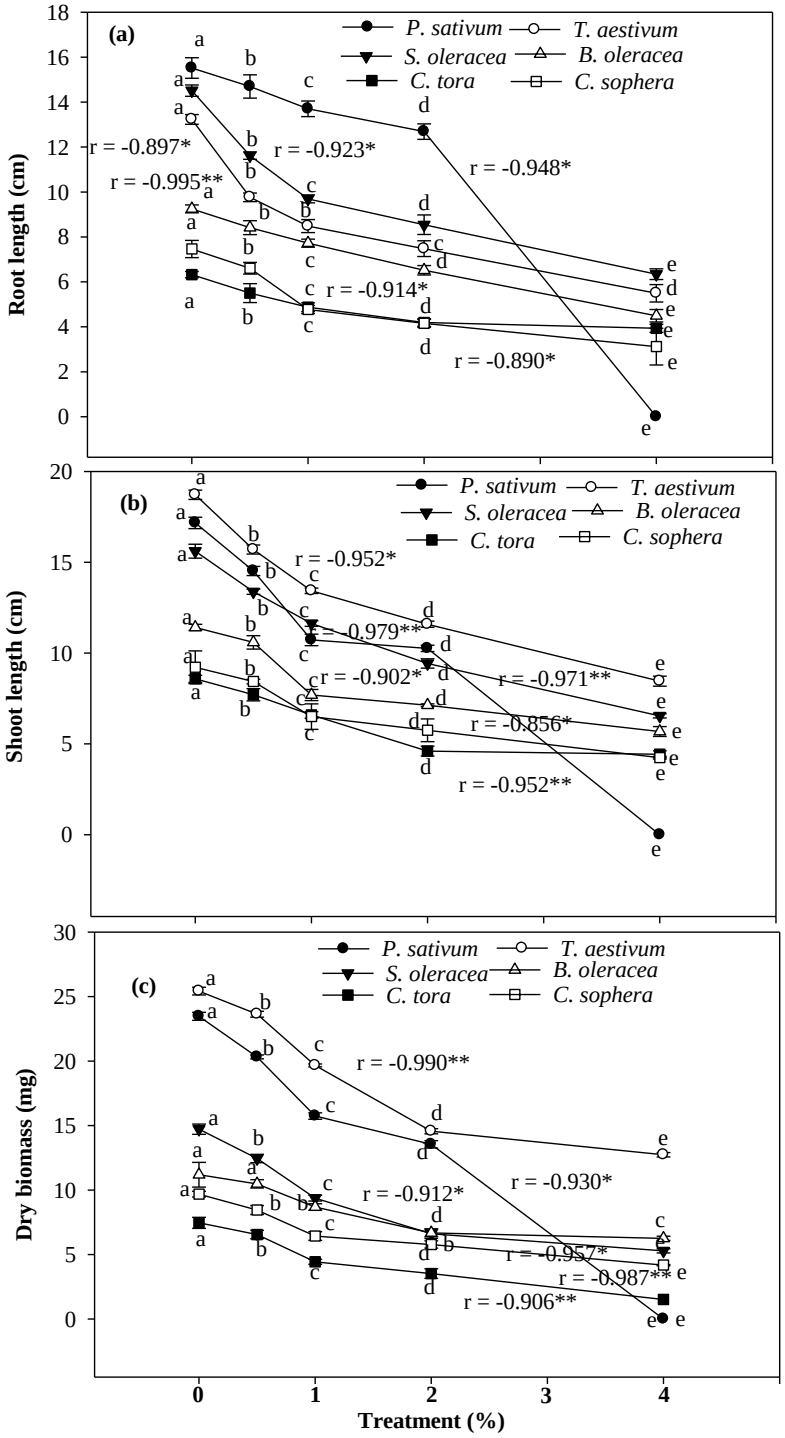
5.4.1.1. Root length

The root length of the test plants (crop and weed) under investigation when grown in aqueous extract of leaves, were lesser than that of control. In control, the maximum growth was seen in *P. sativum* (15.51±0.45 cm) followed by *S. oleracea* (14.51±0.25 cm), (13.23±0.21 cm) in *T. aestivum* and (9.24±0.17 cm) in *B. oleracea* var. *botrytis* but in weed plants, root length was (7.46±0.38 cm) in *C. sophera* and (6.20±0.48 cm) in *C. tora*. When the set-up was subjected to the aqueous extract of leaf, the lengths of the roots were measured to be relatively shorter than their respective control (Fig. 2.1a). In case of all these test plants, reduction in root length was increased with increasing concentration while it was maximum (100%) in *p. sativum* with no seeds germinated at 4% and minimum (37.80%) in *C. tora* at 4%. In all these test plants, reduction was measured to be above 38%. The values of correlation coefficients between root length and concentration of aqueous extract of leaves were strong with values ranging from -0.890 to -0.995.

5.4.1.2. Shoot length

The shoot length of crop plants was measured to be maximum (18.71±0.25 cm) in *T. aestivum* followed by that of *P. sativum* (17.6±0.31 cm), *S. oleracea* (15.06±0.38 cm) and *B. oleracea* var. *botrytis* (11.41±0.16 cm) (Fig. 2.1b) while in weed plants, maximum shoot length observed was in *C. sophera* (9.22±0.90 cm) followed by *C. tora* (8.58±0.19 cm), respectively in control. When test plants were grown in aqueous extract of leaf, the plumule length was shorter than their respective values in control. The very strong effect was seen at highest concentration (4%) and among all the test plants about 48.36% to 100% reduction were observed. The values of correlation coefficient between shoot length and concentration of aqueous extract of leaves were strong in each of the cases. The correlation coefficient values ranged from -0.902 to -0.990.

Fig. 2.1: Allelopathic effect of leaf aqueous extract of *C. procera* at flowering stage on (a) root length (b) shoot length and (c) dry biomass of recipient species (crops and weeds).



Significant difference is represented by different superscript symbols among themselves along a curve at $p<0.05$ applying DMRT.

r represents correlation coefficient.

* and ** represent significance of correlation at $p < 0.05$ and $p<0.0$, respectively.

5.4.1.3. Dry biomass

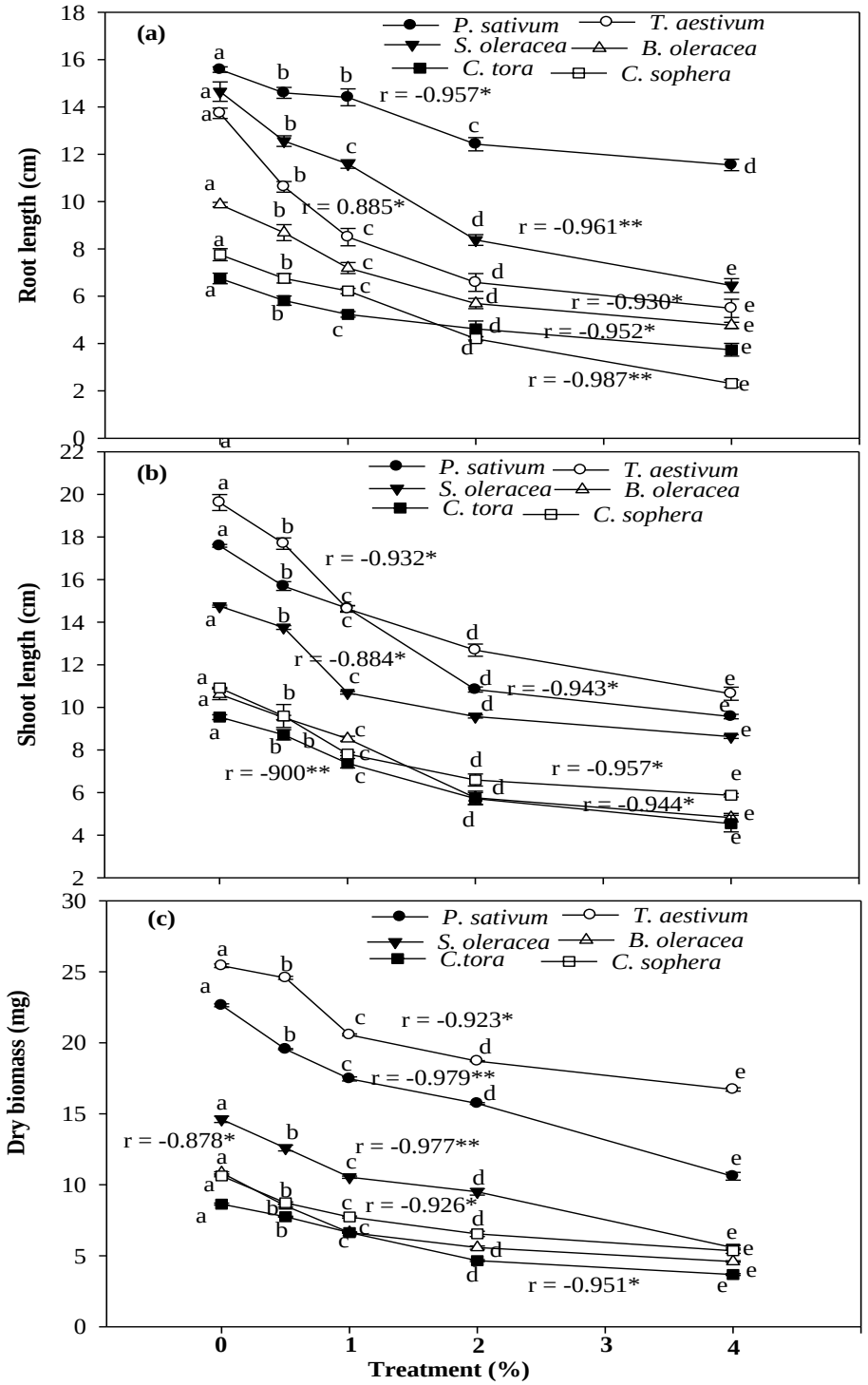
Maximum content of dry biomass was noticed in case of crop plants (25.42±0.30 mg/seedling) in *T. aestivum* followed by *P. sativum* (23.46±0.31 mg), *S. oleracea* (14.71±0.39 mg) and *B. oleracea* var. *botrytis* (11.18±0.95 mg). In weed plants, the maximum dry weight was seen in the case of *C. sophera* (9.68±0.22 mg) and *C. tora* (7.45±0.42 mg). In contrast to the control-grown plants, those grown in aqueous extract of leaf, the dry biomass was relatively less. The reduction was observed in all test plants about 40.52% to 100% at 4% concentration (Fig. 2.1c). The values of the correlation coefficient between the concentration of aqueous extract of leaves and dry biomass were strong and reciprocal in all the test plants ranging from -0.906 to -0.990.

5.5. Stem aqueous extract prepared from *C. procera* and its effect

5.5.1. Root length

In response to extracts from the stem, the seedling root length of all test plants (crops as well as weeds) decreased with increasing concentration. In case of crops, maximum root length was seen in *P. sativum* (15.57±0.11 cm) followed by *T. aestivum* (13.73±0.71 cm), *S. oleracea* (14.64±0.60 cm) and *B. oleracea* var. *botrytis* (9.87±0.09 cm) while maximum growth was observed in *C. sophera* (7.75±0.25 cm) followed by *C. tora* (6.75±0.22 cm) in case of weed plants grown with pure water (control). In control, among the six test plants under investigation, *P. sativum* have the longest roots and contrary to this, the shortest root length was seen in *C. tora*. In comparison, of these, those grown in aqueous extract of stem, root length were measured to be shorter than that of their respective control. With every increase in concentration of aqueous extract, the root length was seen to be decreased and the differences between the treatments were statistically significant. The effect of extracts among all test plants was noticed at highest concentration, i.e. 4%, maximum reduction was seen in *C. sophera* which reduced nearly (70.19%) and minimum in *P. sativum* nearly (25.88%) while in *T. aestivum, S. oleracea, B. oleracea* var. *botrytis* and *C. tora*, it was reduced by 60.01%, 55.94%, 51.67% and 44.79%, respectively (Fig. 2.2a). The values of the correlation coefficient between the root length and concentration of aqueous extract of stem were strong, ranging from -0.885 to -0.987.

Fig. 2.2: Allelopathic effect of stem aqueous extract of *C. procera* at flowering stage on (a) root length (b) shoot length and (c) dry biomass of test plants (crops and weeds).



Significant difference is represented by different superscript symbols among themselves along a curve at $p<0.05$ applying DMRT.
r represents correlation coefficient.
* and ** represent significance of correlation at $p<0.05$ and $p<0.01$, respectively.

5.5.2. Shoot length

The lengths of shoot that emerge from the seeds treated with aqueous extract of the stem were small compared to their respective control. Among the six test plants grown in control, maximum length was seen in *T. aestivum* (19.61±0.37 cm) and shortest length in *C. tora* (9.54±0.11 cm). However, like root length, the shoot length of plants treated with aqueous extract of the stem was shorter compared to the respective values of control (Fig. 2.2b). Although, with every increasing concentration of the extract applied, the length was seen to be decreased from 0.5% to 4% in all test plants. The maximum reduction in shoot length was seen in *B. oleracea* var. *botrytis* reduced by 54.57% and minimum (41.55%) in *S. oleracea.* In case of *P. sativum* and *S. oleracea*, reduction was found to be (45.62% and 41.55%) at the highest concentration. The value of the correlation coefficient in all the cases was reciprocal and strong with value ranging from -0.844 to -0.957.

5.5.3. Dry biomass

The dry biomass of all test plants varied appreciably. In control, the maximum dry biomass (25.43±0.13 mg/seedling) were seen in *T. aestivum* followed by *P. sativum* (22.64±0.10 mg), *S. oleracea* (14.60±0.22 mg) and *B. oleracea* var. *botrytis* (10.84±0.07 mg) while it was also maximum in *C. sophera* (10.62±0.05 mg) followed by *C. tora* (8.64± 0.07 mg) in weed plants. Like seedling growth, the dry biomass of test plants were less than their respective values of the control (Fig. 2.2c). Among all test plants, maximum effect on dry biomass was observed in *S. oleracea* (5.57±0.15 cm) the reduction was 61.84% and lowest in *T. aestivum* (34.29%) at highest concentration compared to the respective value of the control. While in case of *B. oleracea* var. *botrytis* and *C. tora*, dry biomass was reduced by almost same value (57.65%). In all six cases, correlation coefficient values between dry biomass and concentration of aqueous extract were strong ranging from -0.878 to -0.979.

5.6. Effect of aqueous extract prepared from roots of *C. procera*

5.6.1. Root Length

In crop plants, *P. sativum* was measured to have the root length (15.96±0.02 cm) followed by *T. aestivum* (14.10±0.10 cm), *S. oleracea* (13.74±0.26 cm) and *B. oleracea* var. *botrytis* (9.95±0.04 cm). In weed plants, maximum root length was measured to be (7.68±0.45 cm) in *C. sophera* followed by *C. tora* (7.05 ±0.05 cm) in control. When seeds of six test plants were subjected to growth trial in Petri dishes to

study the effect of aqueous extract derived from the roots of *C. procera.*, the root length of test plants was less than that of control (Fig. 2.3a). The reduction ranged from 27.58% to 43.81% in the case of crops and in *C. tora and C. sophera*, it was reduced by 27.73% and 40.14%. Among all cases, the root length was decreased with increasing concentration. The correlation coefficient values between root length and aqueous extract of root were seen to be strong, negative and reciprocal with values ranging from -0.883 to -0.994.

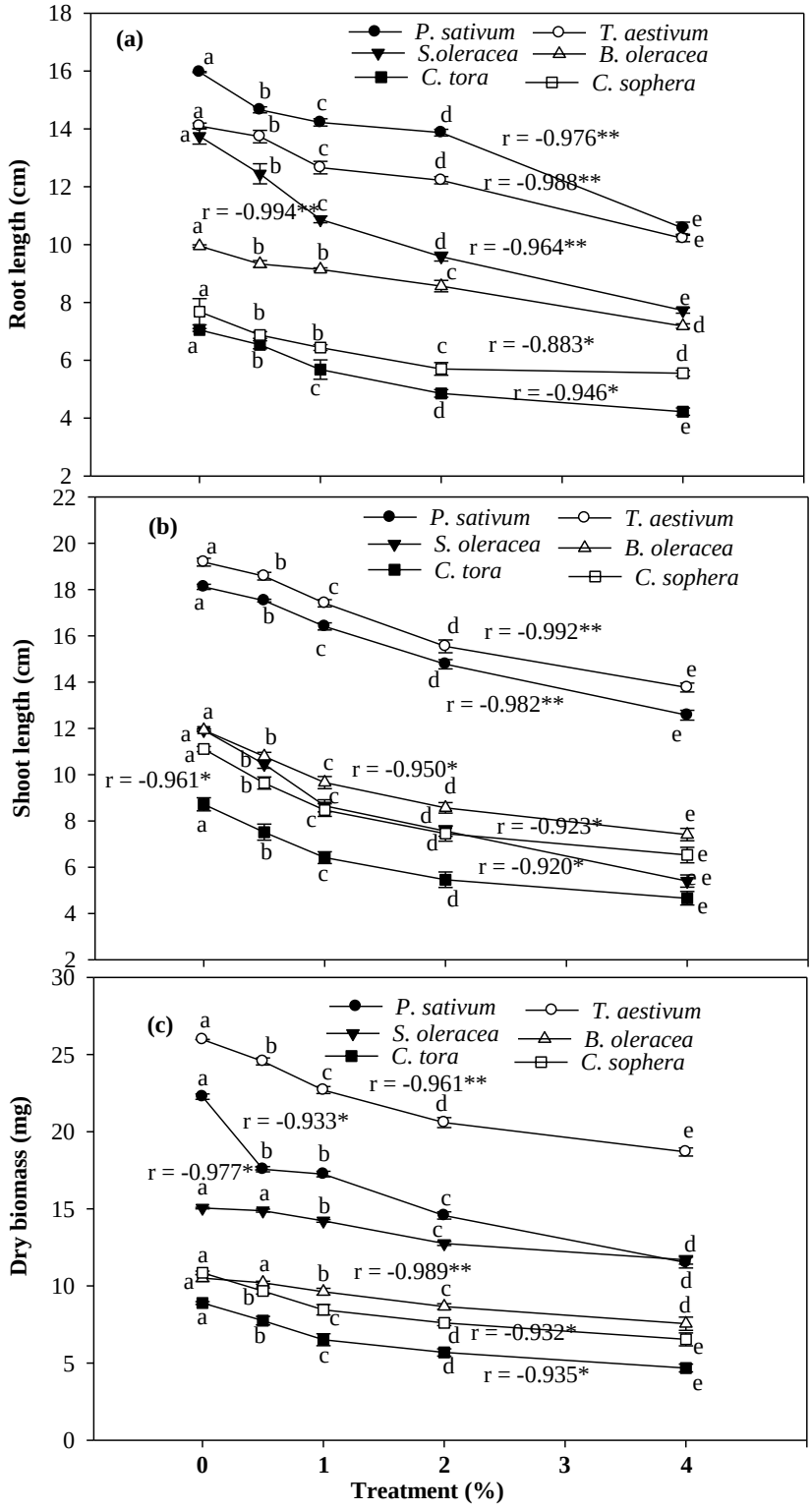
5.6.2. Shoot Length

The shoot length of *T. aestivum* and *P. sativum* seeds sown in control (pure water) were (19.18±0.16 cm) and (18.11±0.11 cm), respectively. The maximum effect was observed in *B. oleracea* var. *botrytis* with the increasing concentrations of aqueous extract of root. In comparison to the others (crop and weed plants) the maximum reduction was measured to be (54.69%) in *S. oleracea* at 4% (Fig. 2.3b). The highest concentration of aqueous extract of the root was more effective for the test plants. In *B. oleracea* var. *botrytis*, reduction of (37.91%) was observed, followed by (30.64%) in *P. sativum* and (28.25%) in *T. aestivum* while in weed plant (41.26%) reduction in *C. sophera* was noticed at 4%. In all test plants the values of correlation coefficient representing a degree of correlation between the two and varies ranging from -0.920 to -0.992.

5.6.3. Dry Biomass

The dry biomass was maximum in *T. aestivum* (25.79±0.01 mg/seedling) followed by *P. sativum* (22.26±0.17 mg), *S. oleracea* (15.05±0.05 mg) and *B. oleracea* var. *botrytis* (10.50±0.22 mg) while in *C. sophera*, it was (10.85±0.11 mg) and *C. tora* (8.89±0.09 mg) in the control set. It was observed that the maximum dry biomass noticed was in *T. aestivum* (18.68±0.26 mg) and the minimum was in *C. tora* (4.68±0.25 mg) at 4% concentration. The content of dry biomass was decreased with increasing concentration in all the test plants. The reduction of the percentage was above 23% in each case (Fig. 2.3c). The values of correlation coefficient were calculated with value ranging from -0.932 to -0.989.

Fig. 2.3: Allelopathic effect of root aqueous extract of *C. procera* at flowering stage on (a) root length (b) shoot length and (c) dry biomass of test plants (crops and weeds).



Significant difference is represented by different superscript symbols among themselves along a curve at $p<0.05$ applying DMRT.
r represents correlation coefficient.
* and ** represent significance of correlation at $p<0.05$ and $p<0.01$, respectively.

5.7. EFFECT OF LEAF AQUEOUS EXTRACT ON MORPHOLOGICAL AND FOLIAR MICROMORPHOLOGY

As per our results, the leaf aqueous extract seems more inhibitory than the root and stem extract. In addition to the reduction in seedling growth and dry biomass, the treated seedling of *P. sativum* also shows visual symptoms in the form of darkening and rottening (Plate 2.1). The *P. sativum* seedlings showed the pronounced rottening and darkening of seedlings along with the thinning and darkening of root tip at their respective concentrations. The seedlings of *C. sophera* showed distinguished morphological abnormality such as negative gravitropism (Plate 2.2) in addition to reduced root and shoot lengths, inhibition of primary root growth and non-emergence of primary leaves from the cotyledon at 4% treatment. Only *P. sativum* and *C. sophera* with their effect on morphology have been taken, this is due to the more allelopathic impact of leaf aqueous extract on these in comparison to other species.

SEM analysis revealed the different alteration of leaf surface of *C. sophera* along with the formation of major grooves, canal and cyst like structures on exposure to leaf aqueous extract in contrast to control (Plate 2.3). The leaf surface was more or less smooth and did not exhibit damaged epidermal cells or ruptures in the control treatment. However, on exposure to 0.5% and 1% treatment, contraction and shrinkage of epidermal cells were noticed losing their stability. The cells become wrinkled and shrinked. However, in response to 2% concentration, the injury level was very different and the cells formed canal like structures. Further, the canal structures become more apparent with the formation of cavities on the leaf surface and the formation of cyst on the leaf surface on exposure to 4% treatment.

5.8. EFFECT ON pH, OSMOTIC POTENTIAL AND TOTAL PHENOLIC CONTENT OF EXTRACT PREPARED FROM DIFFERENT PARTS OF C. PROCERA

5.8.1. pH

Although the pH of the extract differed within different extract concentration, the study didn't reveal much change in the pH of the extract. The near neutral pH was observed with values ranging from 6.66 to 6.90 in extracts (i.e. leaves, root and stem) (Table 2.1). The statistical significant difference in the pH of root, stem and leaf extracts was observed.

5.8.2. Osmotic potential (OP)

Further, the osmotic potential of extracts prepared from the different parts (leaves, stem and root) was also measured. The values of OP ranged from -0.046 bars to -0.096 bars (Table 2.1) and it was observed that the OP increased with increasing concentrations and was statistically significant.

5.8.3. Phenolic content

Extracts prepared from different parts in different concentrations at flowering stage, the amount of phenolics was also determined. The amount of phenolic was about (828.06±14.42 µg/ml) at 4% concentration in leaf extract. The maximum content of phenolics was depicted in the leaf extract followed by root and stem extract. The difference between extract and concentration was statistically significant (Table 2.1).

Table 2.1: Values of pH, osmotic potential and content of phenolic in different concentration of extracts of leaves, stem and roots of C. *procera.*

Parameters	Extract concentration	Stem	Root	Leaf
	0.5	6.33±0.035^{d}	6.48±0.060^{b}	6.66±0.051^{c}
pH	1.0	6.41±0.047^{c}	6.49±0.032^{b}	6.52±0.041^{c}
	2.0	6.53±0.047^{b}	6.62±0.058^{a}	6.42±0.060^{b}
	4.0	6.62±0.026^{a}	6.70±0.015^{a}	6.95±0.015^{a}
LSD at 5%		**0.13**	**0.15**	**0.14**
	0.5	-0.33±0.05^{c}	-0.36±0.05^{c}	-0.46±0.05^{c}
Osmotic potential (bars)	1.0	-0.36±0.05^{c}	-0.46±0.05b^{c}	-0.79±0.05b^{c}
	2.0	-0.53±0.04^{b}	-0.56±0.05^{b}	-0.88±0.05^{ab}
	4.0	-0.73±0.03^{a}	-0.80±0.10^{a}	-0.96±0.04^{a}
LSD at 5%		**0.015**	**0.019**	**0.018**
	0.5	73.82±15.39^{c}	264.10±44.69^{c}	499.13±78.15^{b}
Total phenolic content (µg/ml)	1.0	96.82±5.80^{b}	300.04±136.65^{ab}	512.30±17.78^{b}
	2.0	147.57±31.31^{b}	444.80±40.80^{bc}	532.46±46.88^{b}
	4.0	248.49±47.80^{a}	536.83±55.73^{a}	828.06±14.42^{a}
LSD at 5%		**98.81**	**265.03**	**156.12**

Different alphabets within a column represent significant difference at P<0.05.
± represents standard deviation.

5.9. DISCUSSION

From the results, it is shown that different parts of *C. procera* exhibited phytotoxic potential through their aqueous extracts, though the magnitude of phytotoxicity varied with plant part (i.e. leaves, root and stem). Not only the allelopathic activity of the weed, change with plant part but also with the concentration as noticed in the present study. Based on this observation, phytotoxicity of the weed could be minimized during its heavy infestation in the croplands. Depending on the concentration of the extract, target species and plant tissues from which chemicals are extracted, allelopathic activity varies (Sodaeizadeh *et al.,* 2009; Gulzar and Siddiqui, 2013ab). According to previous reports, diaspore germination and seedling growth was inhibited upon exposure with increasing concentration of extracts (Ashrafi *et al.,* 2009, Zhang *et al*., 2010a). Wandscheer and Pastorini (2008) and Grisi *et al*. (2012) reported that extracts were more active at higher concentrations by analyzing the allelopathic effect of leaves and roots of *Raphanus raphanistrum* L.

on diaspores and seedlings of lettuce and tomato, as was also observed in the present study. Similar results have been recorded for other species of donor plants (Povh *et al*., 2007; Wandscheer and Pastorini, 2008; Scrivanti, 2010; Souza *et al.,* 2010; Grisi *et al*., 2012). Our study therefore, revealed that some growth inhibitors are present in the extract that might be affecting the growth of the test plant. The phytotoxic nature of aqueous extract of weed in nature that reduced the growth of other plants have been indicated by a number of studies (Qasem and Foy, 2001; Bulut *et al*., 2006; Gulzar and Siddiqui, 2013b; Gulzar and Siddiqui, 2014a,b,c; Gulzar *et al.,* 2014a,b,c; Gulzar *et al*., 2015a; Gulzar and Siddiqui, 2016). The Phytotoxic/allelopathic effect of aqueous extract of weeds has been reported by some recent studies that include *Cannabis sativa* (Pudelko *et al*., 2014), *Bothriochloa laguroides* var. *laguroides* (Scrivanti, 2010), *Trigonella foenum-graecum* (Omezzine *et al.,* 2014a,b), *Achillea biebersteinii* Afan. (Abu-Romman, 2011), A*geratum conyzoides* (Jayaraman and Ramalingam, 2014), *Amaranthus retroflexus*, *Chenopodium album*, *Erigeron canadensis* and *Solanum nigrum* (Marinov-Serafimov, 2010), *Amaranthus hybridus* (Amini and Ghanepour, 2013), *Cassia sophera* (Gulzar *et al.,* 2014a), *Cassia tora* (Sarkar *et al.,* 2012), *Chenopodium album* (Majeed *et al.,* 2012), *Chenopodium album*, *Amaranthus retroflexus* and *Cynodon dactylon* (Rezaie and Yarnia, 2009), *Chenopodium murale* and *Malva parviflora* (Al-johani *et al.,* 2012), *Chrysanthemoids monilifera* ssp. *monilifera* (Al Harun *et al.,* 2014), *Cleome arabica* and *Capparis spinosa* (Ladhari *et al.,* 2014), *Coronopus didymus* (Khaliq *et al.,* 2013b), *Cymbopogon nardus* (Suwitchayanon and Kato-Noguchi, 2014), *Euphorbia guyoniana* (Nasrine *et al.,* 2013), *Heracleum sosnowskyi* (Balezentiene and Renco, 2014) and *Salvia plebia (*Husna *et al.*, 2016).

The observed phytotoxicity of *C. procera* may be attributed to the presence of variable amounts of phytotoxic substances in different parts that leach out under natural conditions (Khan and Musharaf, 2012) supports our results. As per reports of (Xuan *et al*., 2004; Ahmad, 2012), foliar leachates have been regarded to be most phytotoxic in nature (probably owing to their proportionately greater biomass and with greater metabolic activity or production of more metabolites. Although, allelopathic activity may be contributed by each organ of the plant. However, the leaf is the most metabolically active plant organs, with higher concentration (Sodaeizadeh *et al.,* 2009) and diversity of allelochemicals (Ribeiro *et al.,* 2009, Tanveer *et al.,*

2010; Grisi *et al*., 2012). Gatti *et al*. (2004) and Wu *et al.* (2009), stated that the greater allelopathic effect of *Aristolochia esperanzae* Kuntze and *Mikania micrantha* Kunth is due to leaf extracts than their respective root extracts on the germination process of target species. As discussed by (Dorning and Cipollini, 2006; Wu *et al*., 2009) the difference in the allelopathic effects of the *Sapindus saponaria* root and mature leaf extracts may be related to the different concentrations of allelochemicals or chemical composition among the extracts. Accordingly, control of weeds can be significantly promoted by the stronger allelopathic activity of the leaves. Therefore, for the development of natural herbicides, the leaf of *C. procera* can be considered as an important organ with active principles. Studies have also indicated that phytotoxicity of leachable allelochemicals is dependent upon several factors such as concentration, flux rate, age and metabolic stage of part and environmental conditions (Rice, 1984; Wyman-Simpson *et al*., 1991; Wardle *et al*., 1993; Weidenhamer, 1996).

The observed morphological changes corresponds well to other authors (Olson and McKercher, 1985; Chon *et al.*, 2002; Pudelko *et al.*, 2014) who observed the increased number of seminal roots, reduced lateral root production, decreased root extension, caused root tips to swell (club-like appearance) and negative geotropism after trifluralin application on wheat and stunted and swollen root of alfalfa by exposure to alfalfa aqueous leaf extract at 30 g l^{-1} and coumarin at 10^{-3} M tips. The SEM studies of *Cassia* leaf surface revealed that leaf aqueous treatment caused foliar ultramorphological changes when compared with the control (Gulzar *et al*., 2015a). The SEM analysis of the leaf surface revealed disruption of epidermal cells in the form of canals and formation of cyst like structures instead of being smooth as depicted in the control treatment. These observations are confirmed by a similar findings reporting epidermal cell morphology alteration in *Arabidiopsis thaliana* on exposure to monoterpenes allelochemicals viz; camphor and menthol (Schulz *et al*., 2007).

Considering that the reduction in the germinability of test species was due specifically to the presence of substances with allelopathic activity in the extracts of *C. procera* that leach out in water solutions as the osmotic potential of -0.046 to -0.096 bars and pH of extracts ranged from 6.66 to 6.95 did not interfere with the germination of donor species which is in agreement with the study of Grisi *et al.*

(2012). The pH did not seem to be responsible for the observed effect when the control and extracts were not significantly different as per study of Lorenza *et al.* (2011). However, the pH effect could not be totally excluded when they were different, because inadequate pH can provoke abiotic stress in terrestrial plants (Pedrol *et al.,* 2006; Lorenza *et al.*, 2011). With regard to ion concentration values, these corresponded to concentrations that did not exceed the threshold for phytotoxicity (Abrol *et al.,* 1988; Lorenza *et al.*, 2011). The evaluation of pH and the molar concentration of aqueous extracts is important given that osmotically active substances such as sugars and amino acids that influence the ion concentration (Ferreira and Aquila, 2000) may affect the results of germination and seedling growth (Gatti *et al*., 2004). Phenolics are the most common water-soluble allelochemicals known to play a significant role in plant-plant interactions, including allelopathy (Appel, 1993; Blum *et al.,* 1999; Mizutani, 1999; Batish *et al*., 2002). In order to find out the nature of these growth-inhibiting substances, the amount of total phenolics in the extracts was determined as these are most common water soluble group of allelochemicals playing an important role in allelopathy (Appel, 1993; Mizutani, 1999). An appreciable amount of phenolics was determined in all the extracts and their amount increased with extract concentrations. Further, the amount of phenolics also correlated with the phytotoxic effect of the weed part (although no correlation analysis was made, it is apparent from the data that phytotoxic effect was more where the amount of phenolics was more).

Therefore, on the basis of this observation following conclusion can be made:

- Different parts of *C. procera* exhibit differential phytotoxicity and the degree of phytotoxicity with respect to plant was in the order:

  Leaves > Roots > Stems

- Leaves being more in biomass per plant contributed relatively more towards phytotoxicity compared to other parts of the plant.
- Presence of phenolics imparted the allelopathic/phytotoxic property to the different parts as evidenced from their amount and degree of inhibition of test plants.

SECTION III

Residue Amended Soil

6. OBJECTIVE

To study the phytotoxicity of naturally dried above ground plant material (referred to as residue) of *Calotropis procera* (Ait.) R. Br. on the growth and establishment of some crops and weeds vis-a-vis related changes in the physico-chemical properties of the soil.

6.1. OBSERVATION PARAMETERS

The following observations were made:

1. Estimation of the residue per unit area.
2. Phytotoxicity of residue amended soil (RS), residue extract amended soil (RES) and the residue extract (RE) towards some crops namely *Spinacia oleracea* L., *Brassica oleracea* var. *botrytis* L. and weeds namely *Cannabis sativa* L., *Chenopodium album* L. in terms of root length, shoot length and dry biomass.
3. Dynamics of the release of phenolics in residue-amended soil, residue extract amended soil and residue extract.
4. Physico-chemical properties like pH, electrical conductivity, total water soluble phenolic acids, available macro-(N, P, K, Na, Ca, Mg, Cl, and HCO_3) and micro nutrient-(Zn, Fe, Mn, Cu) in residue amended as well as residue extract amended-soils.
5. Elemental analysis (macro-nutrients-C, H, N, P, K, Na, Ca. Mg and micro nutrients-Zn, Fe, Mn and Cu) of the residue.

6.2. MATERIALS AND METHODS

6.2.1. Collection of the material

C. procera infested site was selected in and around the campus of Aligarh Muslim University, Aligarh. Plant density and biomass were measured by laying 20 quadrats of 1 m^2 each when the plants were completely dried after the completion of its life cycle. The naturally dried plant residue (above ground) was collected, powdered and packed in polyethylene bags for further use.

Soil was collected from an open area free from *C. procera*. It was air dried, sieved through 2 mm mesh and made the lots of 1 kg each.

Crop seeds, namely *S. oleracea* and *B. oleracea var.* botrytis were procured from IARI, New Delhi and weed plants, namely *C. album* and *C. sativa* from NRCWS, Jabalpur (M.P).

6.2.2. Preparation of residue amended and residue extract amended soil

Under natural conditions, *C. procera* upon death falls on the soil floor and gets mixed up there in it. In order to stimulate these conditions 5, 10, 20, 40 g of the residues were added in 1 kg soil lot separately and thoroughly mixed so as to get 0.5%,1%,2% and 4% residue amended soils. For the preparation of residue extract amended soils, firstly, residue extracts were prepared. For this, 40 g powdered residue was immersed in pure water for 20 hours at room temperature. It was filtered through double layer of muslin cloth followed by Whatman filter paper no. 1 to get 4% residue extract. Further dilutions with pure water were done so as to have 2%,1% and 0.5% solutions. These were referred to as residue extracts. In 27×15 cm rectangular plastic trays, 500 ml of each of 0.5%,1%,2% and 4% residue extract was added in 1 kg soil, separately and placed them for drying under shade for 30 hours. After that, 250 g each of the respective residue amended or residue extract amended soil was taken in 15 cm diameter Petri dishes.

The untreated soil was also taken in 15 cm diameter Petri dishes to serve as control. The residue amended soil has been referred as RS and residue extract amended soil and unamended soil as RES and US, respectively.

6.2.3. Growth studies in amended soils

Seeds of *S. oleracea, B. oleracea* var. *botrytis, C. album* and *C. sativa* were used for growth studies. Twenty uniform seeds of each were sown in 'RS' and 'RES' filled Petri dishes (15 cm diameter) along with untreated soil, served as control. For each treatment, five replicates were maintained in a completely randomized block design and placed in a chamber maintained at 25±1°C, 75±3% RH and 16/8 hour light/dark photoperiod. Each Petri dish was sprayed daily with 25 ml water. After 8 days, seedlings were carefully uprooted ensuring minimal damage to the roots. Root and shoot lengths of five seedlings from each Petri dish were measured and their biomass determined after oven drying at 80°C for 24 hours.

6.2.4. Preparation of residue extract and growth studies under laboratory conditions

For preparation of the extracts, 4 g dried residue was dipped in 100 ml of pure water for 20 hours at room temperature. It was filtered through a double layer muslin cloth, followed by Whatman no.1 filter paper to get 4% concentration. Further dilutions were made to get the concentrations of 2%,1% and 0.5%. Total phenolic

content, pH, electrical conductivity of these extracts was measured. The effect of different residue extract concentrations on the growth and establishment of above mentioned test plants was studied under laboratory conditions. Twenty seeds of each test plants were treated with respective extracts. Seeds treated with pure water served as control. The treated seeds were spread out in 15 cm diameter Petri dishes. Each Petri dish was lined with sterilized absorbent cotton wads and over lined with Whatman no. 1 filter paper. Each wad moistened with 15 ml of the respective treatment solution, ensuring no air trapping in the bed. Five replicates of each treatment were maintained in a completely randomized design. The set-up was put in seed germinator maintained at 25±3°C and 75±3% RH. After 8 days (when no more seed germination), lengths of roots and shoots of five uniform seedlings in each Petri dish were measured and dry biomass was determined after oven drying.

6.2.5. Estimation of phenolics from aqueous extracts of *C. procera* residue and in amended soils (RS and RES)

Total phenolics were estimated in four different lots. In the first lot, 500 ml of 4% residue extract was added in 1 kg of dried soil. In the second lot, 40 g residue and 500 ml of pure water was added in 1 kg soil and in the third lot, 500 ml of pure water was added in 1 kg soil and thoroughly mixed. Five grams of the soil were removed from each lot after 4, 8, 12, 16, 20, 24, 30, 36, 48, 60, 72, 96, 120 and 144 hours and then air dried and subjected to extraction of phenolic acids following the method of Swain and Hillis (1959) using folin-ciocalteu reagent. In the fourth lot, 40 g residue was added in one litre pure water. Five ml of residue extract was removed after 1, 2, 4, 12, 16, 20, 24, 30, 36, 48 and up to 60 hours and then phenolics were estimated from each of the residue extracts. Five replicates were maintained from each treatment.

6.2.6. Determination of physico-chemical characteristic of amended soils

Amended soils namely residue amended (RS), residue extract amended (RES) and unamended soils (US or Control) were analyzed for pH, electrical conductivity, organic matter and available macro and micro-nutrients (Batish *et al*., 2002; 2004). The pH and electrical conductivity were measured with digital pH and conductivity meter from the soil paste in pure water in the ratio of 1:5 (w/v) by immersing the electrode in each of it and total phenolic content by Swain and Hillis (1959). Organic carbon and organic matter were measured by using rapid titration method developed

by (Walkley and Black, 1934; Batish *et al*., 2002; 2004). Available nitrogen was also estimated by following AOAC, 1960 using alkaline $KMnO_4$ and available phosphorus by the method of Olson *et al.* (1954) using an ammonium molybdenum solution, whereas the estimation of available potassium and sodium by following the method of Bower and Gschwend (1952), While available calcium and magnesium by versenate (EDTA) method and available chlorides and bicarbonates were determined by the titration method. For the estimation of micro-nutrients, diethylene triamine penta acetic acid (DTPA) was used. For the extraction and the content of these micro-nutrients in the extracted solution were analyzed on an Atomic Absorption Spectrophotometer (AAS).

6.2.7. Elemental analysis of residue

Elements like carbon, nitrogen and hydrogen in the residue were determined using a CHN analyzer and for the analysis of P, K, Na, Ca, Mg and trace elements, wet diacid digestion of the residue was done using nitric acid and perchloric acid while phosphorus was estimated from the plant material duly digested (referred to as plant digest) by colorimetric method using vandamolybdate reagent. Na was determined through flame photometry and Ca and Mg in plant digest by the titration method while Zn, Cu, Fe and Mg concentration in plant digest were determined by Atomic Absorption Spectrophotometer (AAS).

6.3. STATISTICAL ANALYSIS

All the experiments were laid out in a completely randomized block design. The data of root length, shoot length and dry biomass were expressed with respect to control and analyzed by DMRT at $P<0.05$. The results obtained from nutrient analysis were also subjected to DMRT as per Duncan (1955) and two-sample-t-test. The values of correlation coefficients between concentration and respective parameters were also calculated.

6.4. RESULTS

6.4.1. GROWTH STUDIES

6.4.1.1. Growth studies in RS

The seed germination of all test plants, namely *S. oleracea*, *B. oleracea* var. *botrytis*, *C. album* and *C. sativa* in control as well as in the treatments were observed and found that it was 100% so that the data have not been tabulated and presented.

6.4.1.2. Root length

The root length seen to be largest in *B. oleracea* var. *botrytis* (12.55±0.33 cm) followed by *C. sativa* (10.63±0.22 cm), *S. oleracea* (10.41±0.33 cm) and *C. album* (8.86±0.09 cm) when sown in residue free soil (control) while, a significant reduction in its length was observed in residue soil (Fig. 3.1a). The maximum reduction was observed in *C. sativa* (55.12%) followed by *B. oleracea* var. *botrytis* (54.18%) at 4% concentration, hence the root length decreased with the increasing concentrations, i.e. 0.5% to 4% (Fig. 3.1a). Correlation coefficient values ranging from -0.944 to -0.977 between root length and concentration were calculated.

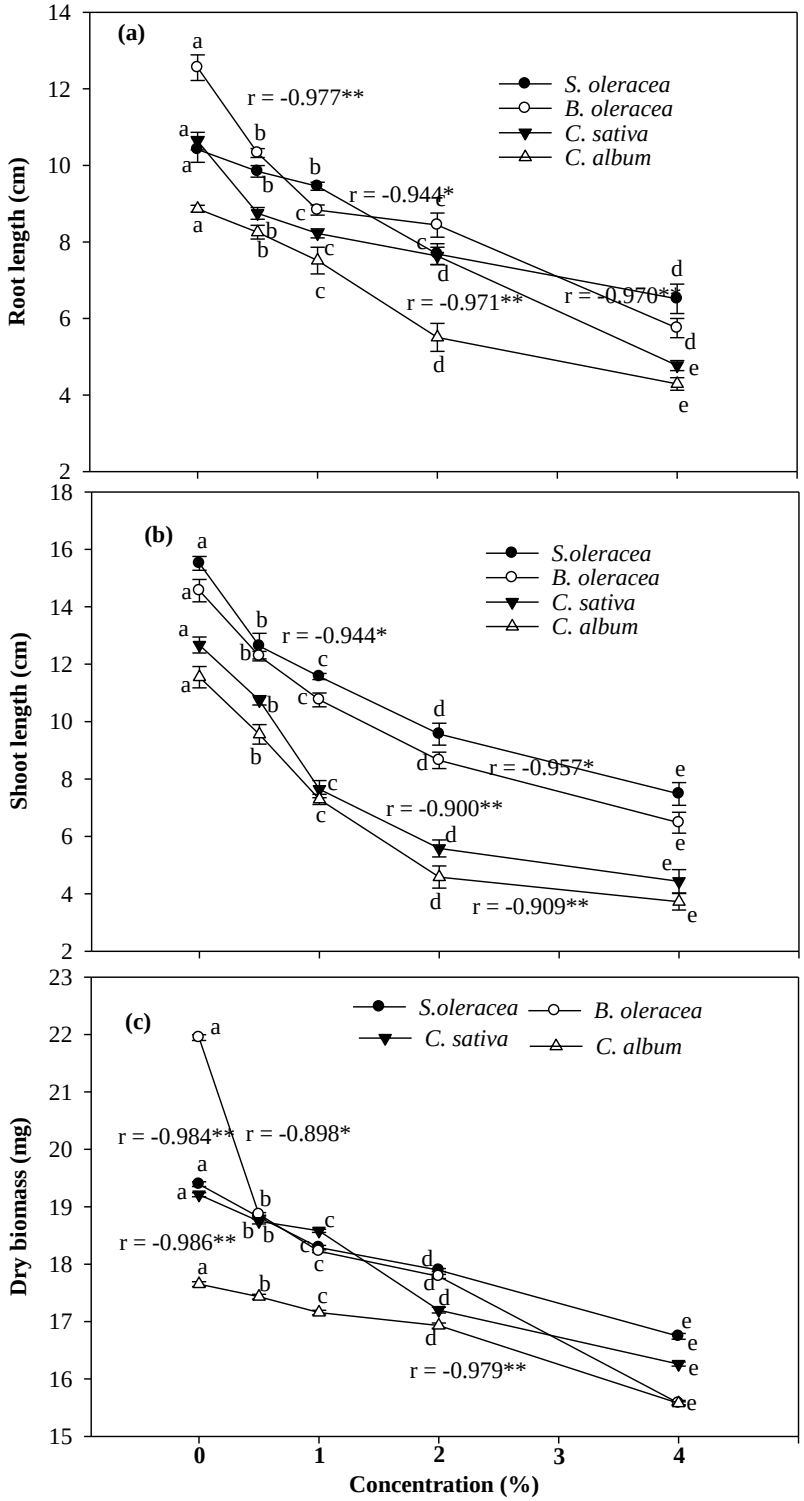
6.4.1.3. Shoot length

The shoot length of test plants varied appreciably, in *S. oleracea* it was (15.51±0.24 cm) whereas in *B. oleracea* var. *botrytis* it was measured to be (14.56±0.38 cm) but in weed plants the shoot length was (12.66±0.27 cm) in *C. sativa* and (11.54±0.37 cm) in *C. album* in control set. The maximum reduction was observed in *C. album* nearly (68%), followed by *C. sativa* (65%), *B. oleracea* var. *botrytis* (55.56%) and *S. oleracea* (51.77%). The reduction in shoot length was statistically significant (Fig. 3.1b). Among all the test plants, shoot length decreased with increasing concentrations of residue amended in soil. Correlation coefficient values between shoot length and concentration of all the test plants were calculated to be strong with values ranging between -0.900 to -0.957.

6.4.1.4. Dry biomass

The maximum dry biomass were observed (21.94±0.05 mg/seedling) in case of *B. oleracea* var. *botrytis* followed by *S. oleracea* (19.39±0.04 mg) and *C. sativa* (19.21±0.03 mg) while the least dry biomass was measured to be (17.65±0.04 mg) in case of *C. album* in control setup (Fig. 3.1c). In all these test plants, maximum retardatory effect was observed at 4% concentration and it was (28.98%) in *B. oleracea* var. *botrytis*, (15.35%) in *C. sativa*, (15.05%) in *S. oleracea* and nearly (12%) in *C. album* (Fig. 3.1c). The dry biomass of all these test plants were decreased with increasing concentration. In all the test plants, the values of correlation coefficients between concentration and dry biomass were reciprocal and relatively strong showing range from -0.898 to -0.986.

Fig. 3.1: Allelotoxic effect of residue amended soil (RS) on (a) root length (b) shoot length and (c) dry biomass of recipient species (crops and weeds).



Significance difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.

r donates correlation coefficient.

* and ** indicates significance of correlation at $p<0.05$ and $p<0.01$, respectively.

6.5. Growth studies in RES

6.5.1. Root length

The growth studies in RES indicated a significant effect. The root length of *B. oleracea* var. *botrytis* was measured to be (11.76±0.19 cm) followed by *C. sativa* (11.11±0.11 cm), *S. oleracea* (10.81±0.15 cm) and *C. album* (7.68±0.20 cm) in control. The maximium effect (2.63±0.22 cm) on root length was seen in *C. album* at 4% concentration over the control (Fig. 3.2a). In case of all test plants, reduction were increased with increasing concentration (0.5% to 4%). The maximum reduction was observed in *C. album* nearly (66%) followed by *C. sativa* nearly (62%) at the highest concentration as compared to control. The minimum effect on root length was observed in *S. oleracea* nearly (33%) in comparison to other test plants. In all the test plants, strong correlation coefficient values were calculated, indicating an almost consistent decrease in root length with increasing concentrations of amendment (Fig. 3.2a).

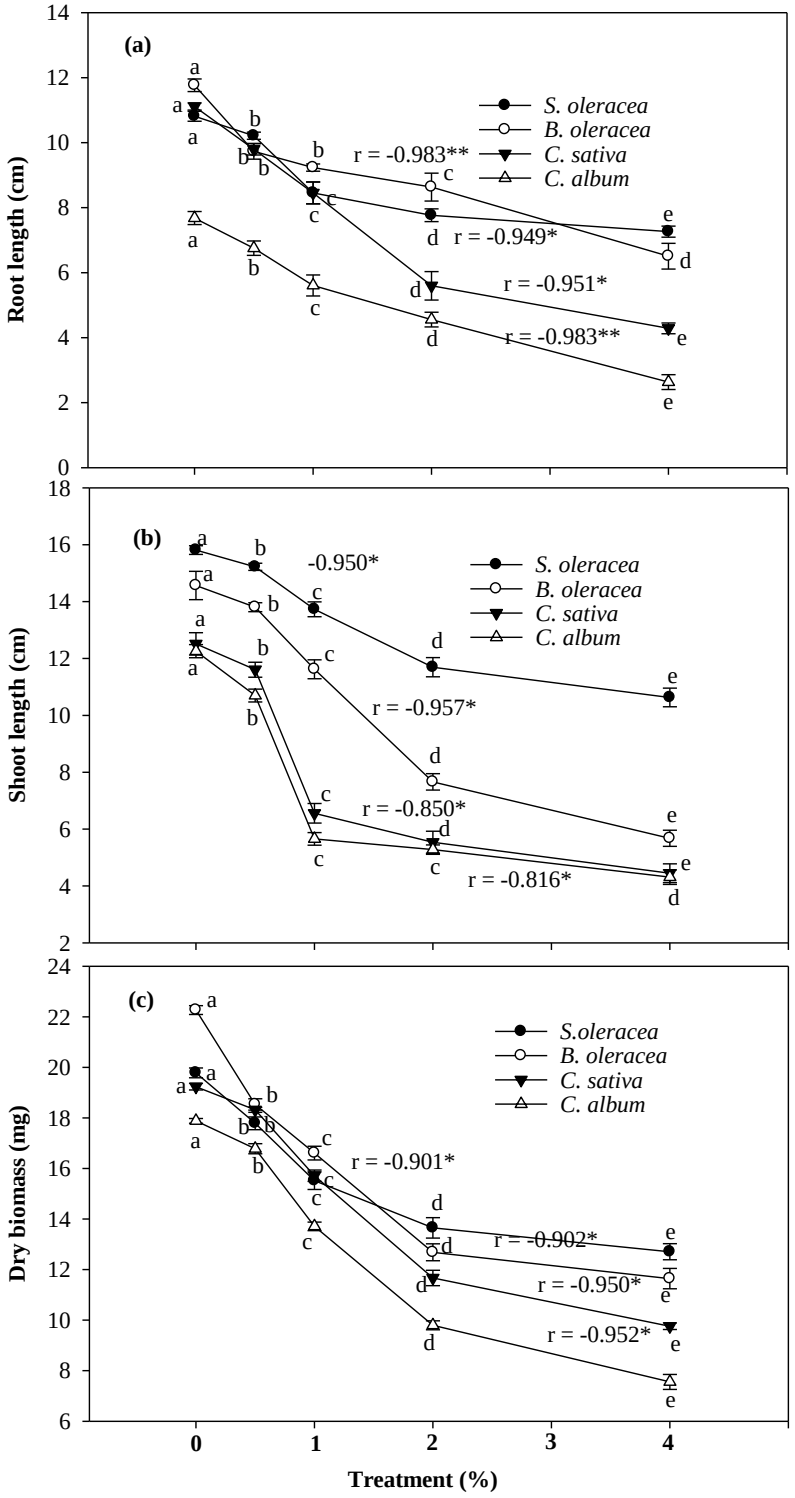
6.5.2. Shoot length

Among test plants under investigation, *S. oleracea* was seen to have the longest shoot length (15.81±0.15 cm) followed by *B. oleracea* var. *botrytis*, *C. sativa* and *C. album* (14.56±0.49 cm), (12.51±0.39 cm) and (12.25±0.23 cm), respectively in control (Fig. 3.2b). The shoot length was seen to be decreased with every increased concentration of the RES and the differences between the successive treatments were statistically significant. At 4% concentration, the maximum and almost similar reduction (65%) was noticed in *C. album* and *C. sativa* whereas in *B. oleracea* var. *botrytis* and *S. oleracea* there was a much difference in reduced value, i.e (61.05%) and (32.76%). The values of the correlation coefficient between the shoot length and concentration were strong ranging from -0.816 to -0.957.

6.5.3. Dry biomass

The *B. oleracea* var. *botrytis* (22.26±0.17 mg/seedling) was having the maximum dry biomass followed by *S. oleracea* (19.78±0.19 mg), *C. sativa* (19.23± 0.13 mg) and the least dry biomass was seen in *C. album* (17.88±0.09 mg) in control set (Fig. 3.2c). In contrast to the amendment free (control) soil grown plants, those grown in residue extract amended soil, the dry biomass was relatively less. Although, with every increase in concentration, there was a significant decrease in biomass. The

Fig. 3.2: Allelotoxic effect of residue extract amended soil (RES) on (a) root length (b) shoot length and (c) dry biomass of recipient species (crops and weeds).



Significance difference at p<0.05 represented by different superscript symbols along a curve among themselves applying DMRT.
r donates correlation coefficient.
* and ** indicates significance of correlation at p <0.05 and p<0.01, respectively.

values of correlation coefficients were strong, reciprocal with values ranging from -0.901 to -0.952.

6.6. Growth studies in RE

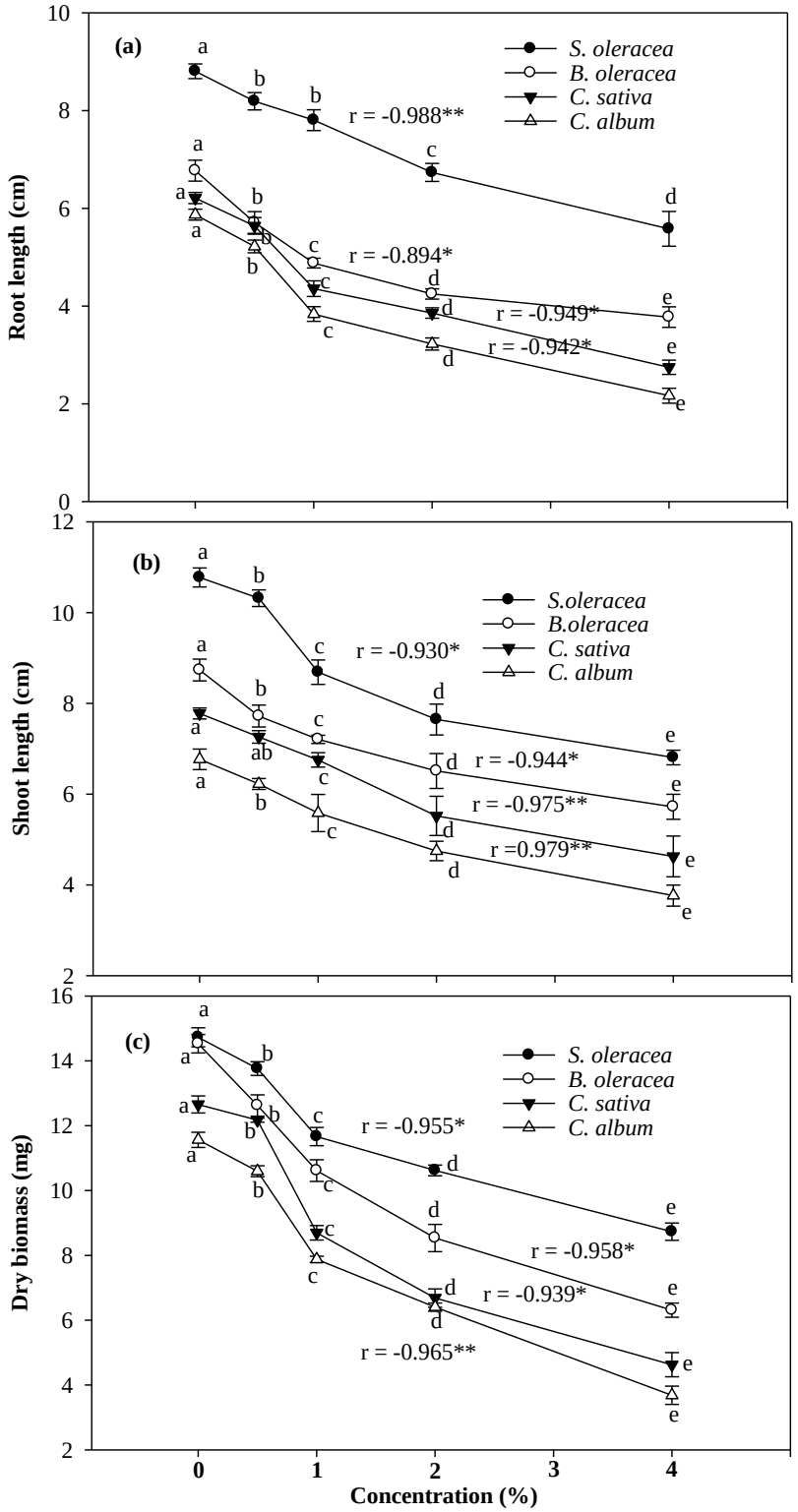
6.6.1. Root length

When the seeds of test plants under investigation were subjected to growth trial in Petri dishes for the study of the effect of aqueous extracts derived from residue, *S. oleracea* and *B. olareacea* var. *botrytis* were measured to have the radicle length of (8.80±0.14 cm) and (6.77±0.21 cm), respectively and was followed by *C. sativa* (6.21±0.11 cm) but least in case of *C. album* (5.87±0.10 cm) in control. However, *S. oleracea* was seen to have maximum radicle length of all germinated seeds sown in Petri dishes of control. In each case of test plants tried, when treated with any of the concentration of the aqueous extract of residue, the root length was shorter in comparison to their respective controls. Further, with every increasing concentration of extracts, the radicle length was seen to be decreased and the trend was similar in almost all the cases (Fig. 3.3a). The values of the correlation coefficient between the radicle length and concentration of aqueous extract were very strong, ranging from -0.894 to -0.988.

6.6.2. Shoot length

The lengths of plumule that emerged from the seeds treated with aqueous extracts of residue were very less compared to their respective control. Among all test plants grown in control, maximum length was seen in the case of *S. oleracea* (10.77±0.21 cm) followed by *B. oleracea* var. *botrytis* (8.73±0.24 cm), *C. sativa* (7.77±0.12 cm) and the shortest was in *C. album* (6.76±0.22 cm). However, like radicle length, the plumule length were also shorter as compared to the respective values of control (Fig. 3.3b). Every increasing concentration of the extract applied, the length was seen to be decreased. In other words, the decrease in length was gradual. In all the cases, the value of correlation coefficient was strong and reciprocal with values from -0.930 to -0.979.

Fig. 3.3: Allelotoxic effect of residue extract (RE) on (a) root length (b) shoot length and (c) dry biomass of recipient species (crops and weeds).



Significance difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.
r donates correlation coefficient.
* and ** indicates significance of correlation at $p<0.05$ and $p<0.01$, respectively.

6.6.3. Dry biomass

Again, the dry biomass of test plants varied appreciably, similar with that of radicle length and plumule length. The dry biomass of *S. oleracea* was measured to be maximum (14.72±0.29 mg/seedling), as compared to other plants and it was followed by *B. oleracea* var. *botrytis* (14.53±0.28 mg) and the least biomass of (11.56±0.23 mg) was seen in case of *C. album* in control set-up (Fig. 3.3c). The dry biomass of plants that emerge from seeds treated with aqueous extracts derived from residue extract were less than their respective values of control (Fig. 3.3c). The reduction in dry weight was statistically significant with increasing concentration and the highest concentration (i.e. 4%) was more effective for *C. sativa* and *C. album*. However, nearly (69%) reduction was observed in *C. album* followed by *C. sativa* nearly (64%). In all the four cases, correlation coefficient values between dry biomass and concentration of aqueous extract was strong with values ranging from -0.939 to -0.965.

6.7. DYNAMIC OF RELEASE OF PHENOLIC CONTENT

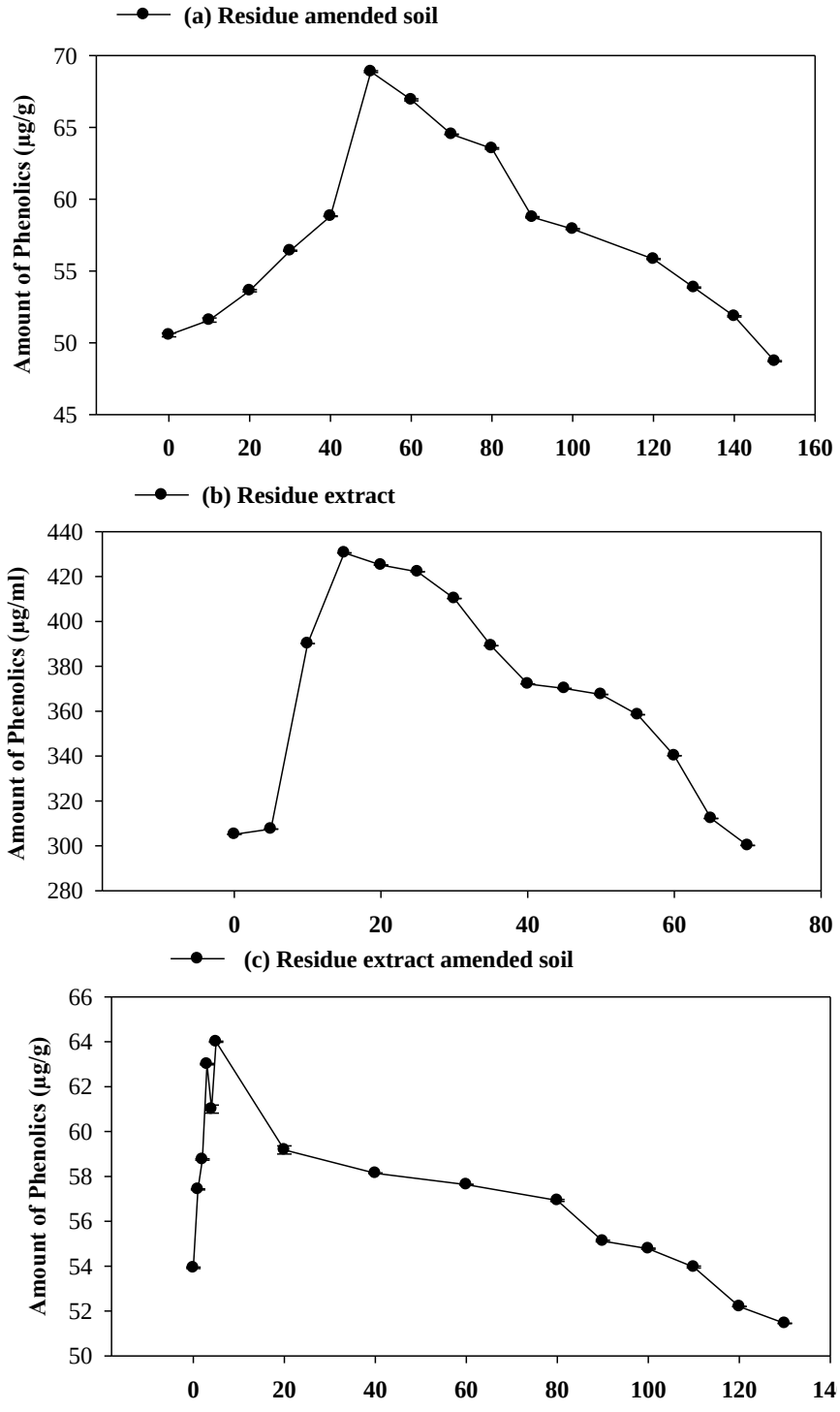
6.7.1. Residue soil (RS)

The amendment free soil (control) was found to contain (51.54±0.13 µg/g dry) weight of phenolic content. The total amount of phenolic content minus phenolic content in control soil is presented in Fig. 3.4a. The maximum amount of phenolic content (68.88±0.06 µg/g) was estimated to be present in amendment at 50 hours. Between zeros to 50 hours, the content showed a gradual increase. After that, a constant decline was, however, noticed from 60 hours onwards with minimum (48.71±0.04 µg/g) recorded at 150 hours.

6.7.2. Residue extract (RE)

It was observed that after 5 hours of adding pure water to the residue, (305.15±0.05 µg/ml) phenolic content was estimated. There was not much difference in the release of phenolics from 0-5 hours. However at 10 hours, the content increased abruptly (390±0.02 µg/ml) with the highest value recorded at 15 hours (430.54±0.04 µg/ml). From 20-30 hours, the decline in phenolic content was gradual. After that, the minimum phenolic content (300.16±0.03 µg/ml) was recorded at 70 hours (Fig. 3.4b)

Fig. 3.4: Dynamics of release of phenolics w.r.t. (a) residue amended soil (b) residue extract and (c) residue extract amended soil.



± represents standard deviation

6.7.3. Residue extract soil (RES)

Based on the above that the maximum amount of phenolic content leached out of the residue after 2 hours in aqueous medium. The suspension of residue in water was filtered after 2 hours and added to the soil mixed thoroughly. The maximum amount of phenolic content (64.0±0.02 µg/g) was measured at 3 hours of the addition of the extracts and after that the value of phenolic content declined. The process of declining was very slow, continued up to 130 hours and it becomes only (51.45±0.01 µg/g) (Fig. 3.4c).

6.8. CHARACTERISTICS OF RESIDUES EXTRACTS

6.8.1. pH

pH of the extracts was found to be near neutral ranging from (6.56±0.06 to 6.71±0.20). It is evident that so much of the change was not reported with the increasing concentration from 0.5% to 4% (Table 3.1).

6.8.2. Electrical conductivity

The electrical conductivity increased with increasing concentration of extracts from (2.61±0.39 µS) to (8.65±0.31 µS) (Table 3.1). This increase was seen to be linear.

6.8.3. Phenolic content

The amount of phenolics in the extract concentration of 0.5% was calculated to be (177.80±28.62 µg/ml) and with increasing concentration of extracts, the value also increased and at 4% concentration, it was nearly 5 times more than in 0.5% (671.17±40.17 µg/ml) (Table 3.1). The increase was also linear, similar to that of electrical conductivity.

Table 3.1: Values of pH, electrical conductivity and phenolic content in residue extracts of *C. procera.**

Treatments	pH	Electrical Conductivity (µS)	Phenolic Content (µg/ml)
0.5	6.71±0.20^{b}	2.61±0.39^{d}	177.80±28.62d
1.0	6.73±1.77^{a}	4.55±0.58^{c}	273.69±17.15c
2.0	6.75±0.049^{b}	5.90±0.37^{b}	470.84±32.36b
4.0	6.56±0.060^{b}	8.65±0.31^{a}	671.14±40.17a
LSD at 5%	**2.97**	**1.42**	**96.30**

Significant difference at $p<0.05$ represented by different superscript symbols among themselves applying DMRT.
± represents standard deviation.
* represents significant difference in values.

6.9. Elemental analysis of the residue

The residues prepared from the aboveground parts of C. *procera* plants were estimated to contain (36.79±0.24%) total carbon, (6.84±0.30%) total hydrogen and (2.15±0.19%) total nitrogen. The residue of *C. procera* when analysed for the available elements, showed (0.26±0.04%) phosphorus, (1.81±0.53%) potassium, (0.10±0.10%) sodium per dry weight (Table 3.2). Macro-nutrients, like calcium and magnesium constituted (38.75±0.28%) and (25.67±0.38%), respectively. Among the micro-nutrient, Fe was maximum with a value of (15.10±0.63 ppm). It was followed by Mn (3.50±0.34 ppm). Zn and Cu were estimated to be (0.74±0.05 ppm) and (0.25±0.01 ppm), respectively, on the dry weight (Table 3.2).

Table 3.2: Content of elements in *C. procera* residue per dry weight.

Element(Units)	**Value**
Total C (%)	36.79±0.24
Total H (%)	6.84±0.30
Total N (%)	2.15±0.19
Available P (%)	0.26^{ns}±0.04
Available K(%)	1.81±0.53
Available Na (%)	0.10^{ns}±0.10
Available Ca (g/100g)	38.75±0.28
Available Mg (g/100g)	25.67±0.38
Available Zn (ppm)	0.74±0.05
Available Cu (ppm)	0.25^{ns}±0.01
Available Fe (ppm)	15.10±0.63
Available Mn (ppm)	3.50±0.34
LSD at 5%	**0.58**

±represents standard deviation; ns- non-significantly different.

6.10. PHYSICO-CHEMICAL PROPERTIES OF AMENDED SOILS

6.10.1. pH

The value of pH decline in the amended soils compared to unamended (control) field soil where it was measured to be (6.92±0.02). In case of RS and RES, the change in pH was insignificant at 0.5%. At the highest concentration, however, pH changed significantly (Fig. 3.5a). The soils in which 4% residue and its extract were amended, the value of pH were measured to be (6.23±0.04) and (6.18±0.01), respectively. The values of correlation coefficients between the concentration of extract/residue amended in soil and the value of pH were strong and reciprocal.

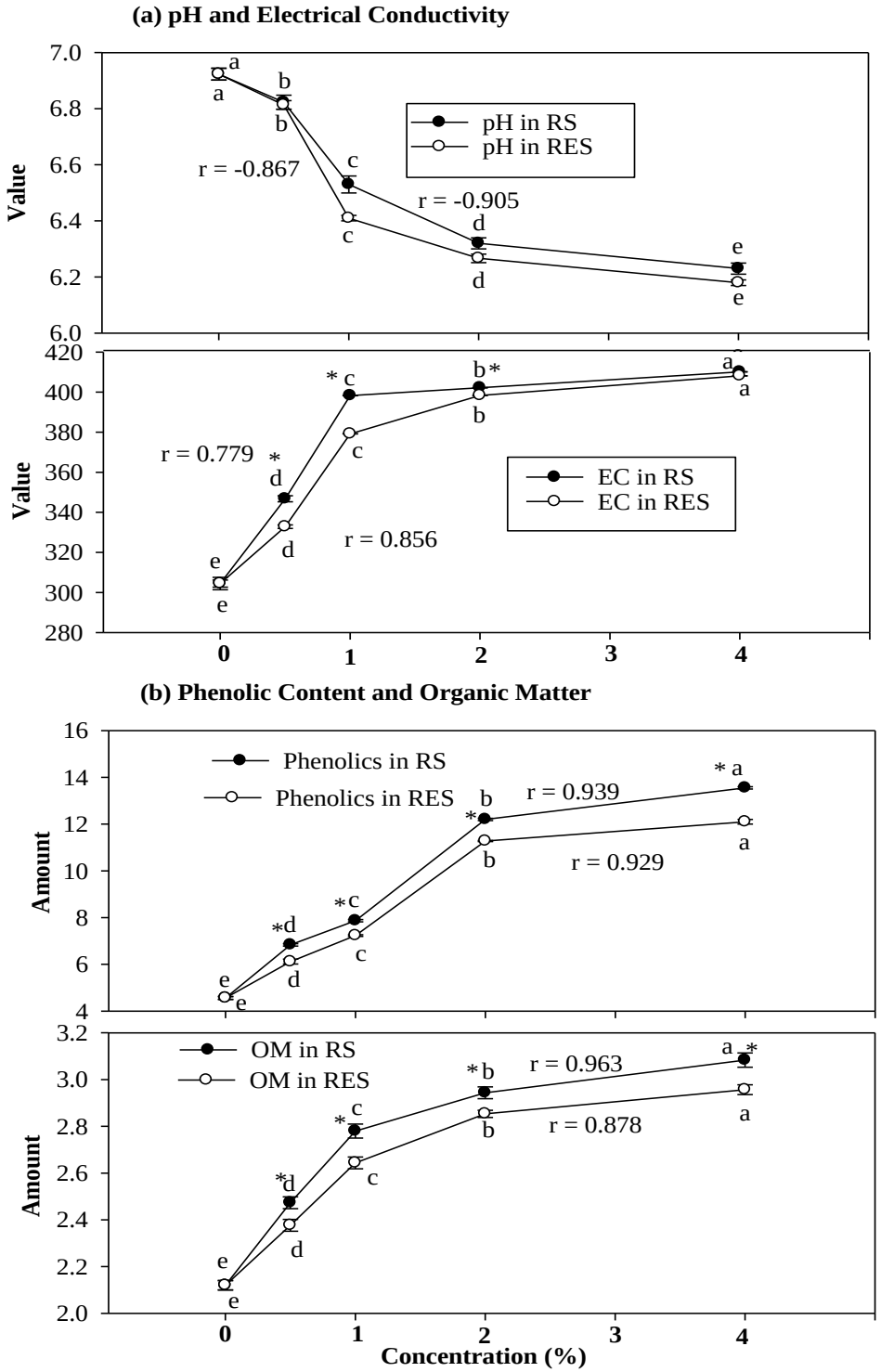
6.10.2. Electrical conductivity

The electrical conductivity of the US was measured to be (304.45±3.07 µS). On addition of the residue or its extracts in the soils, the EC values were seen to be increased with increasing concentration. The increase of EC in RS at the highest concentration of residue, i.e. 4% was nearly (410.12±0.025 µS) compared to US. However, in case of RES, at the same concentration, the increase was 404.12±0.014 µS. While comparing the EC values of RS and RES, the differences were statistically significant except at 4% (Fig. 3.5a). Further, the value of the correlation coefficient in this case was very high and indicating a strong reciprocal correlation.

6.10.3. Phenolic content

The phenolic content in the control soil was (4.56±0.06 mg/100g) of dry weight. In contrast, the amount of phenolic content was significantly more in the amended soil irrespective of the amendment made with extracts or residue (Fig. 3.5b). The content increased with the increasing concentration of amendments. At the highest concentrations of amendment, i.e. 4%, the amount of phenolics increased by (13.55±0.05 mg/100g) and (12.10±0.10 mg/100g) in RS and RES, respectively. The difference in the amount of phenolics at respective concentration of RS and RES was significant (Fig. 3.5b). The values were +0.929 and +0.939 for RS and RES, respectively, reflecting a strong correlation.

Fig. 3.5: (a) pH and electrical conductivity (b) Phenolic content and organic matter in soil amended with different concentrations of residue and residue extract.



Significant difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

6.10.4. Organic matter

The amount of organic matter in the control soil was measured to be (2.12± 0.02%) on dry weight basis. In the RS and RES, the amount of organic matter at 4% concentrations was estimated to be (3.08±0.30%) and (2.95±0.02%), respectively (Fig. 3.5b). The values of the correlation coefficient in this case were also strong +0.963 and +0.878, for RS and RES, respectively. Further, the differences in organic matter in RS and RES at respective concentrations were statistically significant (Fig. 3.5b).

6.10.5. Available Nitrogen and Phosphorus

The available nitrogen in *C. procera* free soil was measured to be (165.55± 0.05 kg/ha). The value of total available nitrogen in RS and RES were statistically insignificant at a concentration of 4% (Fig. 3.6a) and in between these, a statistically significant increase in available nitrogen was observed. At 4% amendment in cases of RS and RES, the amount of nitrogen was (200.23±0.20) and (198.49±7.72 kg/ha), respectively. The correlation values were +0.867 and +0.971 for RS and RES, respectively, reflecting a strong correlation.

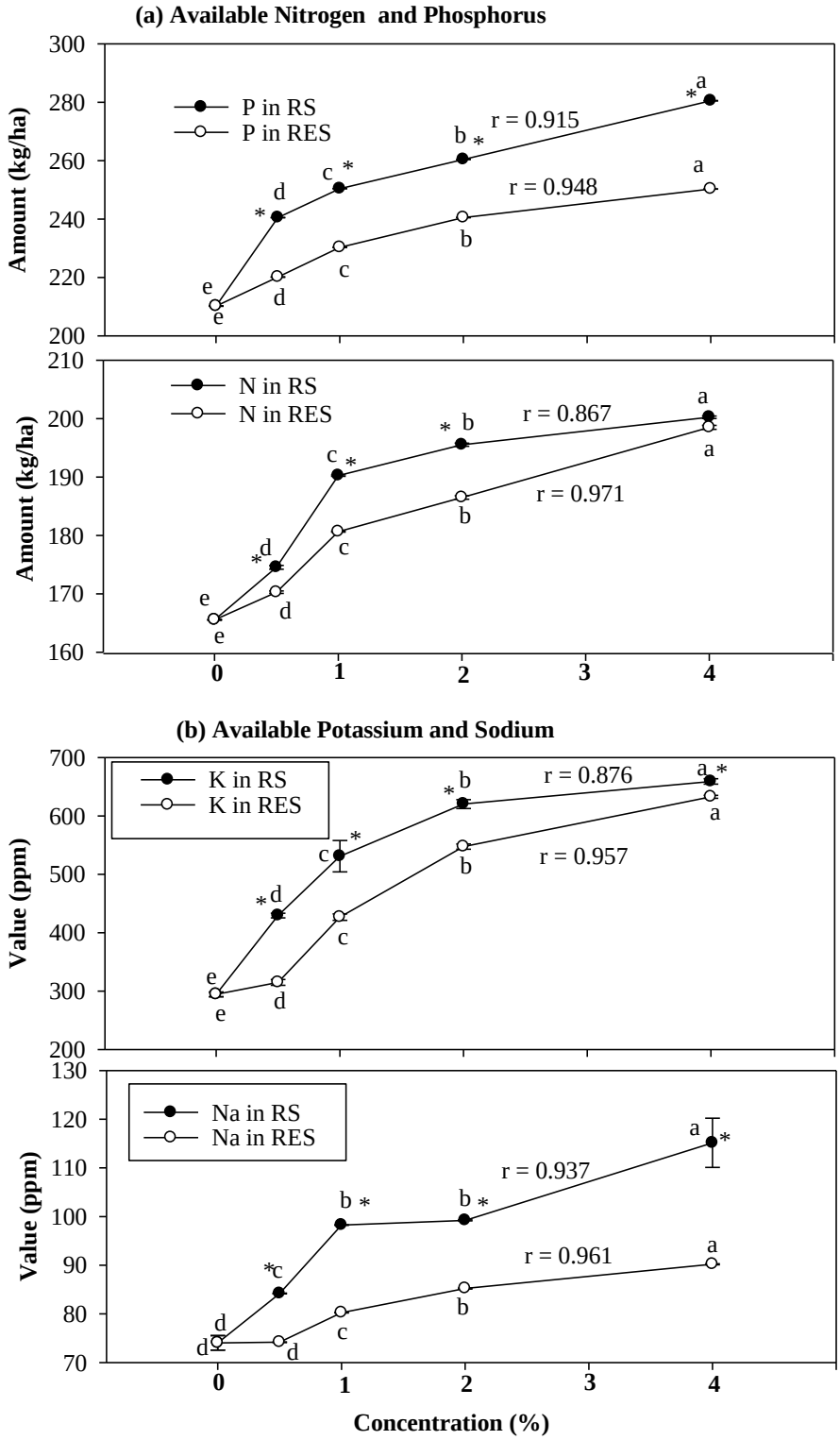
The available phosphorus content in control soil was measured to be (210.20± 0.05 kg/ha) and significant differences were observed at respective concentrations of RS and RES (Fig. 3.6a). At 4% concentration, the amount of phosphorus detected in RS and RES was noticed to be (280.50±0.05 kg/ha) and (250.27±0.03 kg/ha), respectively.

6.10.6. Available Potassium and Sodium

The potassium content in US was (294.33±4.04 ppm) and it increased significantly in the amended soil. With every increase in concentration of RS and RES, the values showed statistical significance. However, the increase in the value of potassium was relatively less in RES compared to RS and found significant at respective concentration. The strong values (+0.957 and +0.876) of correlation coefficient were also calculated for RS and RES (Fig. 3.6b).

The available sodium in control soil was (74.04±1.48 ppm), the trend of changes was similar as that of potassium. The content increases with increasing concentration in RS and RES. However, the dimensions of the change were relatively less, i.e. with every increasing concentration of RES, compared to RS (Fig. 3.6b). The

Fig. 3.6: (a) Available phosphorus and nitrogen (b) Available potassium and sodium in soil amended with different concentrations of residue and residue extract.



Significant difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

difference between the two, i.e. RS and RES at their respective concentrations was highly statistically significant

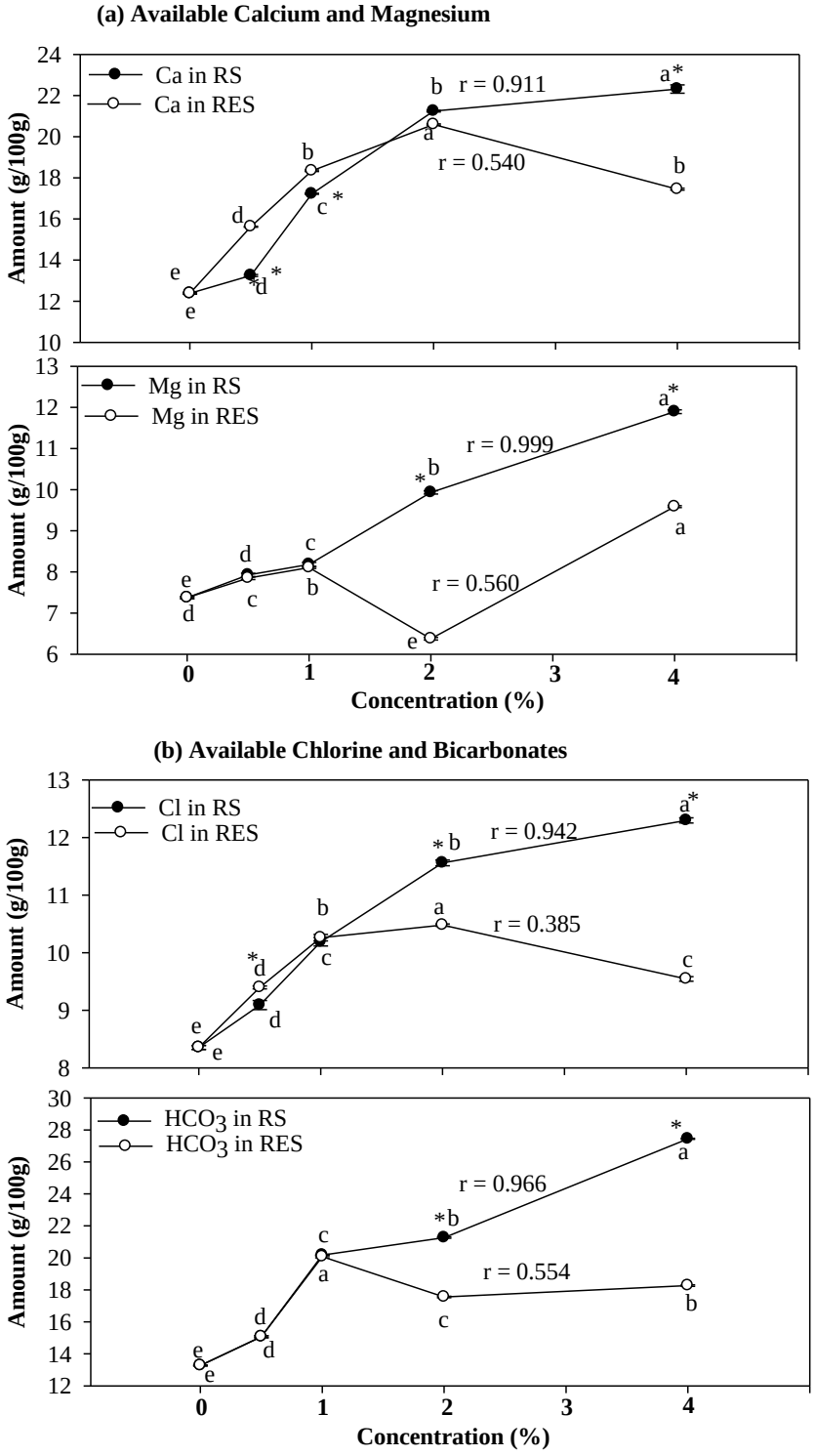
6.10.7. **Available Calcium and Magnesium**

The control soil was measured to have (12.38±0.03 g/100 mg) of the dry weight of calcium and (7.37±0.02 g/100 mg) dry weight of magnesium. With increasing concentration of amendment in RS, the value of Ca and Mg showed a trend towards increase (Fig. 3.7a). The linearity of increase was seen in the case of RS rather than RES and the difference were statistically significant at respective concentrations. However, in RES, at 2% and 4%, the amount of Ca declined. Further, the amount of Mg at 2% declined by the amount of (6.37±0.03 g/100 mg), less than the value of control soil, with sharp increase at 4% in RES. The difference in the amount of Mg was statistically significant at 2% and 4% in RS and RES. It was also apparent from the values of correlation coefficient, with stronger in case of RS (+0.911) compared to RES (+0.540).

6.10.8. Available Chloride and Bicarbonates

The control soil (US) was estimated to possess (8.35±0.03 g/100 mg) and (13.27±0.02 g/100 mg) of chlorides and bicarbonates, respectively (Fig. 3.7b). This increase was seen to be gradual but consistent at least up to 4% in RS and in RES, the contents of chlorine declined at 2% and 4%. In the sample at 4% RS, the content of bicarbonates was measured to be maximum, i.e. (27.44±0.03 g/100 mg). In case of RES, the amount of bicarbonate increased up to 1% and thereafter, it declines, whereas in RS, amount of bicarbonates increased with every increase in concentration of residue (Fig. 3.7b). The difference in the amount of Cl and HCO_3 was statistically significant at 2% and 4%, respectively in RS and RES.

Fig. 3.7: (a) Available calcium and magnesium (b) Available chlorine and bicarbonates in soil amended with different concentrations of residue and residue extract.



Significant difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

6.10.9. Available Micronutrient (Zn, Cu, Fe and Mn)

The amount of Zn, Cu, Fe and Mn was measured to be (3.05±0.05 ppm), (3.52± 0.02 ppm), (13.45±0.05 ppm) and (15.58±0.05 ppm), respectively in unamended (control) soil but in amended soil, however, the amount of microelements increased, by and large, except in a few cases such at a 4% concentration of RES for Cu and Zn. The value of the correlation coefficient between the concentration of RS and RES and content of microelements was strong, (more that 0.8) except in case of Zn and Cu in RES where it was only 0.684 and 0.711 (Fig. 3.8b).

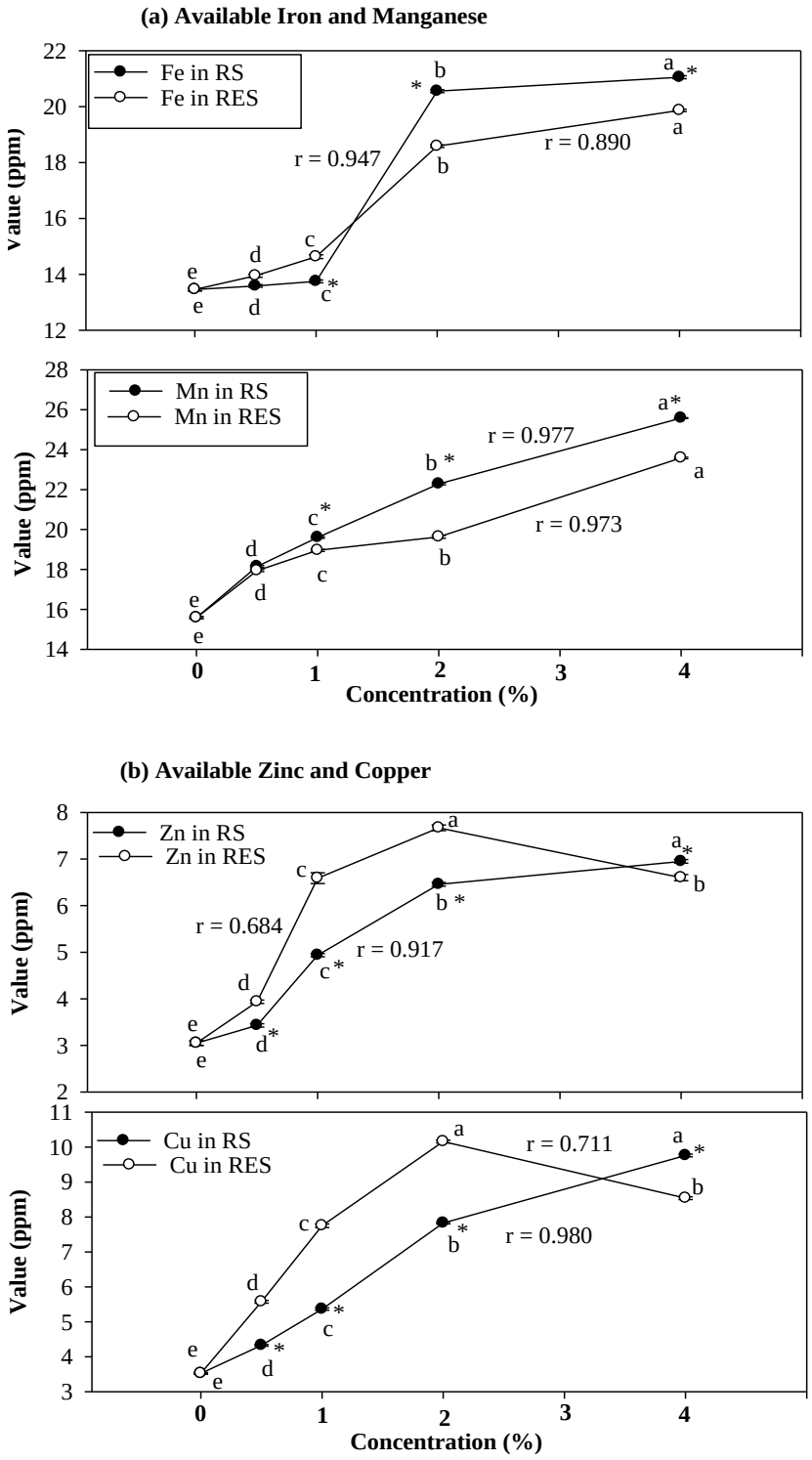
However, the Zn content was more in the RES. It increased with every increasing concentration of residue extract amendation up to 2% and thereafter, it decline. In case of RS, however, a similar trend of increase was observed up to 2% of amendment (Fig. 3.8b). At 4% amendment, little decline in the amount of Zn was observed.

In case of Cu, appreciable change in the amount was observed up to 2% and at 4%, it shows a decline in case of RES. In case of RS, the linear increase in the amount of copper was noticed and besides the contents were more in RES up to 2% than RS (Fig. 3.8b).

In case of Mn, the values in the content of Mn between RS and RES varied significantly after 1% concentration (Fig. 3.8a).

In case of Fe ions, with increasing concentration of amendment by the residue extracts and residue, the abrupt trend towards an increase in the content was noticed at 2% and 4%, with values almost remaining constant at 0.5% and 1% concentration (Fig. 3.8a).

Fig. 3.8: (a) Available iron and manganese (b) Available zinc and copper in soil amended with different concentrations of residue and residue extract.



Significant difference at $p<0.05$ represented by different superscript symbols along a curve among themselves applying DMRT.

* represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

6.11. DISCUSSION

From the present study, large amount of residue of *C. procera* accumulates on the substratum, especially after the completion of its life cycle. Since the plant (*Calotropis*) known to be allelopathic, it is expected that its residue would affect the succeeding vegetation or crops. In order to test this, a number of experiments regarding the growth studies were undertaken under laboratory and greenhouse conditions such as the effect of the extract and amended soils (amended with residue and its extracts). The test plants also carefully chosen since the large amount of residue formation occurs throughout the life cycle of *C. procera.* Thus, the winter season crops like *S. oleracea, B. oleracea* var. *botrytis* and weed plants, namely *C. sativa, C. album* was selected for assessing the phytotoxicity.

Seedling growth and dry biomass were found to be significantly reduced due to the phytotoxic nature of extract prepared from residue in different concentrations compared to control. From the results of the present study, it was found that the growth of the test plants was significantly reduced compared to unamended soil. The test plants exhibited varying degrees of inhibition with maximum retardatory effect noticed in *C. album*. At the highest concentration of extracts, maximum retardatory effect on all the test plants was observed. Thus, this might be due to the allelochemicals that accumulate in soil at bioactive concentration and bring about the inhibitory effect on other plants as the study revealed by Batish *et al*. (2009a). It suggests that residues of *C. procera* contain certain water-soluble phytotoxic principles that upon release accumulate in soil in bioactive concentration and suppress crop and weed growth is in parallel with the study of Batish *et al*. (2009a). Upon incorporation or mixing into the soil, residue undergoes the stage of decomposition and their biomass gradually mixes with the soil that may also be responsible for the inhibitory effect on other test plants. In order to test this, the residue was also amended in the soil and growth of test plants was checked. The growth of all the test plants, especially *C. album* and *C. sativa* were significantly and adversely affected as expected in this setup of experiments. As per reports, even the growth and development of crop plants gets negatively impacted by the incorporation of residues from the invasive weeds ((Batish *et al*., 2006a,b, 2007a,b; Batish *et al.*, 2009a; Yamamoto and Kato-Noguchi, 2015). Similarly, residues (both above and below-ground) of *Ageratium conyzoides* L. significantly reduced the growth, nodule number,

nodule weight and leghemoglobin content on recipient species has been documented by (Batish *et al*., 2004; Batish *et al.* 2006b; Batish *et al*., 2009a). This indicates that different plants show variability in their response with dependence on several factors like size of seeds and genetic differences or variability. The differential response of crops towards extracts, leachates or any other phytotoxic material has already been reported in a number of other crops (Kiemnec and McInnis, 2002; Ambika *et al*., 2003; Xuan *et al.*, 2004).

A number of reports are also available regarding the phytotoxicity of residue extracts indicating that decomposing residue of crops or weeds or even trees release some inhibitors in the environment that may be toxic to the other plants (Chung *et al.*, 1994; Singh, 1996; Prately and Haig, 1997; An *et al.,* 2000a; Singh *et al.*, 2003a,b; Tawaha and Turk, 2003; Belz *et al*., 2007). After the completion of plant life cycle, the residue gets accumulated and comprised dried stem, leaves, part of inflorescence in the case of present study. Due to the allelopathic nature of weed, it can be depicted that allelochemicals are released through various mechanisms such as leachation, death, decay and even exudation (Einhellig, 1988; Singh *et al.*, 2001; Kobayashi, 2004) that bring out the inhibitory effects. Allelochemicals are released through leachation and these are biologically very active becomes evident from the results of the present study. As already reported, the allelochemicals could be either phenolics or sesquiterpene lactones in nature that leach out of the plants (Kanchan, 1975; Mersie and Singh, 1987; Rani, 1990). Therefore, bioefficacy studies were also undertaken in extract amended soil to ascertain whether the inhibitory effect exerted on the test plants is due to the allelochemicals or inhibitors that accumulate in the soil in bioactive concentration after their release through leachation.

In order to find out the allelochemicals or phytochemicals in the extract and amended soil, some specific test such on the presence of phenolics, were conducted. Phenolics are easily leachable and ubiquitous group of allelochemicals in plants and may release through root exudation and decomposition has been indicated by a number of studies (Siqueira *et al.,* 1991; An *et al.,* 1996a,b; 1997; Reigosa *et al.,* 1999b; Kayode, 2006; Srisa-ard, 2007). In our study, a significant high amount of phenolics was found to be present in aqueous extracts of residue. The amount of phenolics was three to four times more in RS and RES soils, respectively, than that in the US that is in line with the study of Batish *et al*. (2009a). The transformation of

phenolics upon entering the complex and heterogenous soil environment could be the reason for a lesser amount of phenolics in RES compared to that in RS (Batish *et al*., 2009a). Phenolics undergo a variety of changes such as sorption or detoxification or even transformed into simpler forms or may serve as a carbon source for microbes (Blum *et al*., 1999; Batish *et al*., 2009a). Further, the available bioactive concentration, quality of phenolics and their influx from the donor plant determine the phytotoxicity of phenolics (Batish *et al*., 2009a). In case of the weed *C. procera*, profuse growth along with its dominance in the infested area and continuous influx of plant residues maintains the availability of phytotoxic compounds into the soil. Amendation of soil with residue/plant material, the change in soil nutrient status, pH and electrical conductivity coincides with an observed growth reduction in most of the allelopathic studies (Harper, 1977; Castells *et al*., 2005; Batish *et al*., 2009a). However, there was no such negative effect of *C. procera* residue amendment on the soil nutrient status as earlier recorded by Batish *et al*. (2009a). Rather, the residue amendment improved the nutrient status of the soil as also indicated by enhanced electrical conductivity and thus greater nutrient availability (Batish *et al*., 2009a). Soil pH was lowered in RS and RES into the soil compared to US. The pH of aqueous extracts was near neutral with increasing concentration. Since the phenolics make the soil acidic, such a lowering of soil pH is not surprising (Dalton *et al*., 1983; Batish *et al*., 2009a). Amount of organic matter and available nutrients was increased in the amended soil and the increase was more in RS than in RES as also revealed by Batish *et al*. (2009a). Increased content of organic matter (Batish *et al*., 2009a) indicates the lesser microbial activity in amended soil (RS and RES) compared to US. It is in line with earlier reports that *C. procera* contain antimicrobial principles (Vadlapudi and Naidu, 2010). Therefore, the possibility of any resource depletion upon residue incorporation and their negative role in causing growth (Batish *et al*., 2009a) has been ruled out in the present study due to observed significant increase in the available nutrient content in RS and RES. The same results were reported from soil incorporation of residues from allelopathic plants by enriching the soil nutrient status rather than depriving it (Batish *et al*., 2002, 2007a; Batish *et al*., 2009a). However, there was a significant reduction in growth of test species in spite of the soil nutrient enrichment in RS and RES due to the phytotoxic phenolic metabolites. Therefore, as per the study of Batish *et al*. (2009a), it indicated seedling growth inhibition might be

due to direct involvement of the phenolics released from residue of *C. procera*. More so, nutrient uptake and transport also get interrupted by these phenolics (Baziramakenga *et al*., 1994) or immobilize the nutrients in soil (Castells *et al*., 2005; Batish *et al*., 2009a) however, we did not explore this aspect in the present study. The dynamics of the release of phenolics in soil were, however, different compared to simple extracts. An appreciable quantity of phenolics was encountered in soil amended with RS, RES and RE as per study of Khaliq *et al*. (2011). Phenolic content showed a periodic increase over time achieving peak (Khaliq *et al*., 2011) at their respective time and after which a decrease in phenolic content was observed in RS, RES and RE. This difference may be due to their relative release from the respective treatments. The composition and quantity of allelochemicals may vary substantially over the time or with changing environmental conditions is in agreement with earlier reports (Wojcik-Wojtkowiak *et al*., 1990; Blum, 1998; Khaliq *et al*., 2011). This confirms the hypothesis by (Kruidhof *et al.*, 2010; Khaliq *et al*., 2011) who proposed that residue-mediated inhibition could occur only if the susceptibility period of the receptor plant coincides with the inhibitory allelopathic potential peak period. For examples, in case of extract amended in soil, the release of phenolics would be relatively easier than from soil residue. Since phenolics are the major category of water-soluble allelochemicals responsible for the most allelopathic activity, their presence was quantified over time after incorporation (Khaliq *et al*., 2011). Thus, the presence of allelochemicals in *C. procera* residue is the primary reason for the growth retardation of test plants.

Thus, the results highlighted in the present section, indicate the following:

1. Appreciable amounts of residue of *C. procera* is accumulated under field conditions, i.e. after the completion of major life cycle.
2. The residue of *C. procera*, like its fresh parts was allelopathic in nature causing a significant retardatory effect on the crops and weeds.
3. Established through this experiment, allelochemicals are released through leachation and decomposition.
4. Based on the observation, the present study concludes that residues of *C. procera* deleteriously affect the early growth of test species by releasing water-soluble phenolic acids into the soil environment and not through depletion of available

soil nutrients as reported by (Batish *et al*., 2009a) in an allelopathic study of *Ageratum conyzoides*.

5. The test plants exhibited varying degrees of inhibition with maximum retardatory effect noticed in *C. album*.

Section IV
Root Residue Amended Soil

7. OBJECTIVE

To study the effect of root residue of *Calotropis procera* (Ait.) R. Br. on some weed and crop plants under laboratory conditions and their impact on the physico-chemical properties of the amended soils.

7.1. OBSERVATION PARAMETERS

The following observations were made:

1. Measurement of roots (length of primary, secondary, tertiary roots and its fresh and dry weight) of *C. procera* per unit area.
2. Phytotoxicity of root residue amended soils (RRS), root residue extract amended soil (RRES) and aqueous extract of root residue (RRE) on some weed and crop plants, namely *Spinacea oleracea* L., *Brassica oleracea* var. *botrytis* L., *Chenopodium album* L. and *Cannabis sativa* L.
3. Dynamics of the release of phenolic content with time intervals in (a) root residue amended soils (b) root residue extract amended soil and (c) aqueous extract of root residue.
4. Estimation of pH, electrical conductivity and phenolic content in aqueous extracts of root residue.
5. Physico-chemical characteristics like pH, electrical conductivity, phenolics, organic matter and available nutrients from root residue amended soil (RRS) and root residue extract amended soils (RRES).
6. Elemental analysis of macro and micro-nutrients of root residue.

7.2. MATERIALS AND METHODS

Experiment was done in the post rainy season to assess the impact of roots of *C. procera*. Four sites at the Aligarh Muslim University campus, Aligarh were marked where *C. procera* was growing abundantly. On these sites, 20 quadrats were made and in each quadrate, observations on a number of the roots/plants, fresh and dry weight of root/unit area were made. The length of primary root, the number and lengths of secondary and tertiary roots were also measured. The roots of all the plants in randomly selected quadrates of $1m^2$ were uprooted by digging method and collected after that, roots were shade dried, powdered and packed in polyethylene bags for further use. Each quadrate served as the sampling unit for measurements of primary, secondary and tertiary roots. The rest of the methodology was similar as given in (Chapter 3, Materials and Methods).

7.3. STATISTICAL ANALYSIS

All the experiments were laid out in a completely randomized block design. The data of root length, shoot length and dry biomass were expressed with respect to control and analyzed by DMRT at $P<0.05$. The results obtained from nutrient analysis were also subjected to DMRT as per Duncan (1955) and two-sample-t-test. The values of correlation coefficients between concentration and respective parameters were also calculated.

7.4. RESULTS

In one square meter of the quadrate, the number of roots of *C. procera* was counted to be (24.44±0.32). The length of primary root was measured to be (22.74±0.23 cm), whereas that of secondary and tertiary roots were (14.32±0.28 cm) and (10.69±0.33 cm), respectively (Table 4.1). The average number of secondary and tertiary roots/root was counted to be (10.24±0.21) and (8.55±0.22). The fresh weight of roots, were found to be (204.43±18.45 g) with their dry biomass measured to be (121.63±6.5 g), respectively (Table 4.1).

Table 4.1: Characteristic features of *C. procera* roots based on quadrate study.

Root Parameters	Values
Primary root (number/m^2)	24.44±0.32
Length of primary roots (cm)	22.74±0.23
Secondary roots (number/m^2)	10.24^{ns}±0.21
Length of secondary roots (cm)	14.32^{ns}±0.28
Tertiary roots (number/m^2)	8.55^{ns}±0.22
Length of tertiary roots (cm)	10.69^{ns}±0.33
Fresh weight of root/m^2 (g)	204.43±18.45
Dry weight of roots/m^2 (g)	121.63±6.5
LSD at 5%	**15.02**

± represent standard deviation; ns non-significantly different.

7.5. GROWTH STUDIES

7.5.1. Growth studies in RRS

7.5.1.1. Root length

When the test plants were grown in unamended (control) soil, the root length of *B. oleracea* var. *botrytis* was measured to be (13.46±0.44 cm) which was slightly longer than the *S. oleracea* ((12.23±0.31 cm), (8.47±0.48 cm) in *C. album* and the least length was noticed in *C. sativa* (5.49±0.34 cm). The root length was reduced with increasing concentration compared to their respective control. This reduction was appreciable and statistically significant at $P<0.05$ (Fig. 4.1a). Consequently, least root length was observed in samples where an amendment was done with 4% root residue concentration. The maximum reduction was measured to be (1.90±0.12 cm) in *C. album* followed by *C. sativa, B. oleracea* var. *botrytis* and *S. oleracea* at 4% concentration. It was reduced by (72.96%) in *C. album,* (65.39%) in *C. sativa,* (51.56%) in *B. oleracea* var. *botrytis* and (30.33%) in *S. oleracea* (Fig. 4.1a). The values of correlation coefficients between root length and concentration in all the test plants were calculated to be strong and reciprocal (more than -0.9 in each case) depicting a strong and negative relationship (Fig. 4.1a).

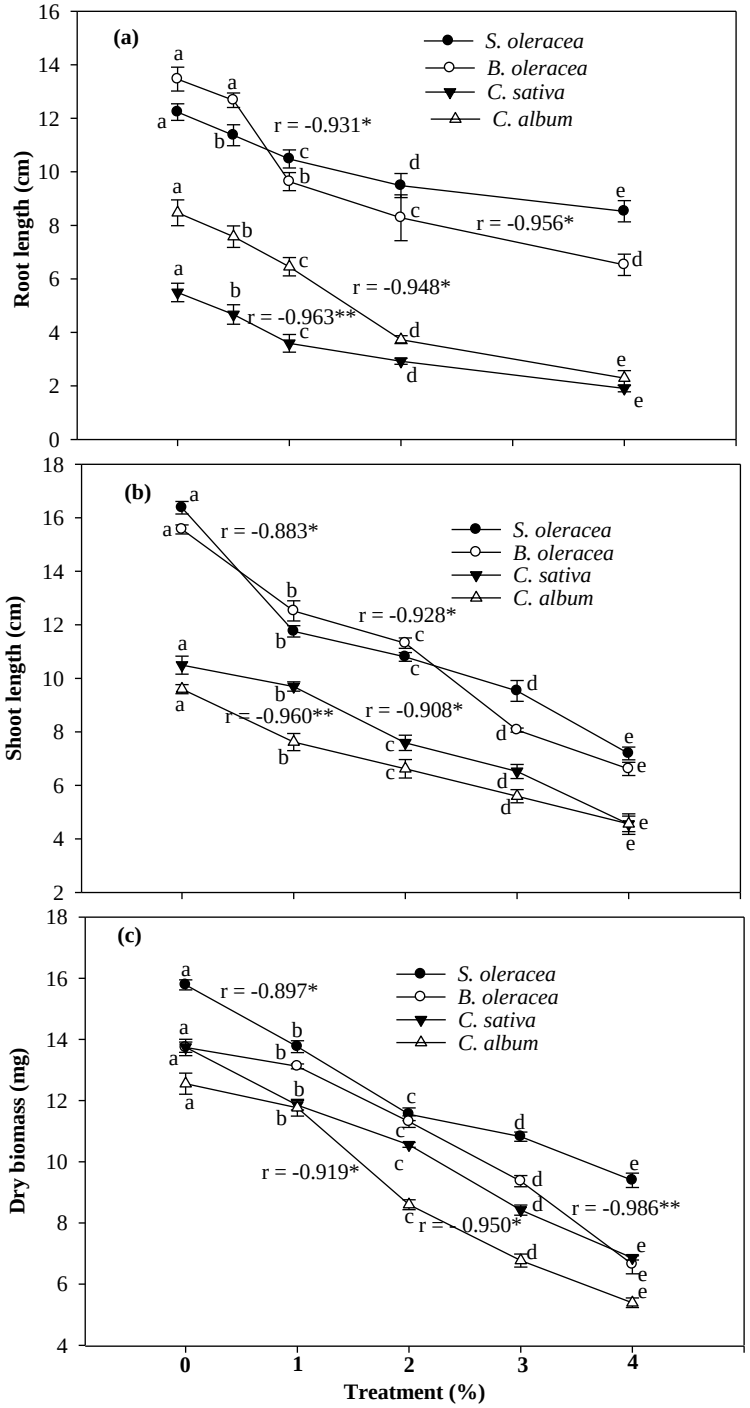
7.5.1.2. Shoot length

The shoot length of *S. oleracea* was maximum (16.37±0.23 cm) in unamended soil (control) followed by *B. oleracea* var. *botrytis* (15.56±0.16 cm), *C. album* (10.49±0.33 cm) and *C. sativa* (9.59±0.16 cm). In contrast to control, shoot length was affected adversely and significantly when grown in root residue amended soils (Fig. 4.1b) and becomes shorter with increasing concentration of amended soil. The correlation coefficient values between shoot length and concentration was calculated to be strong with values ranging from -0.883 to -0.960.

7.5.1.3. Dry biomass

The dry biomass of seedling grown in unamended (control) soil was maximum in the case of *S. oleracea* (15.78±0.16 mg/seedling) followed by *C. album* (13.74 ±0.16 mg), *B. oleracea* var. *botrytis* (13.73±0.26 mg) and *C. sativa* (12.55±0.34 mg). Among all the cases, the minimum dry biomass content was observed to be (5.39±0.15 mg) in *C. album*. The dry biomass of all the test plants, measured to be less when grown in root residue amended soil (RRS) (Fig. 4.1c). This reduction was statistically significant with increasing concentration. The correlation coefficient

Fig. 4.1: Allelopathic effect of root residue amended soil (RRS) on (a) root length (b) shoot length and (c) dry biomass of test plants (crops and weeds).



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.
r represents correlation coefficient.
* and ** represent significance of correlation at $p<0.05$ and $p<0.01$, respectively.

between dry weight and concentration was strong and reciprocal with values ranging from -0.897 to -0.986.

7.6. Growth studies in RRES

7.6.1. Root length

The root lengths of test plants grown in soil amended with root residue extract of *C. procera* were noticed to be decreased with increasing concentrations. In control, maximum root length was measured to be (10.80±0.16 cm) in *S. oleracea* followed by *B. oleracea* var. *botrytis* (8.73±0.25 cm) and (7.74±0.22 cm) in *C. album.* The least root length was observed in *C. sativa* (6.39±0.24 cm) in comparison to other test plants. At 4% concentration of amendment, the maximum reduction in root length was seen in case of *C. album* (64.47%) followed by *S. oleracea* (61.48%) (Fig. 4.2a). The correlation coefficient values between root length and concentration were strong and reciprocal, thereby showing some linearity with values ranging from -0.891 to -0.912.

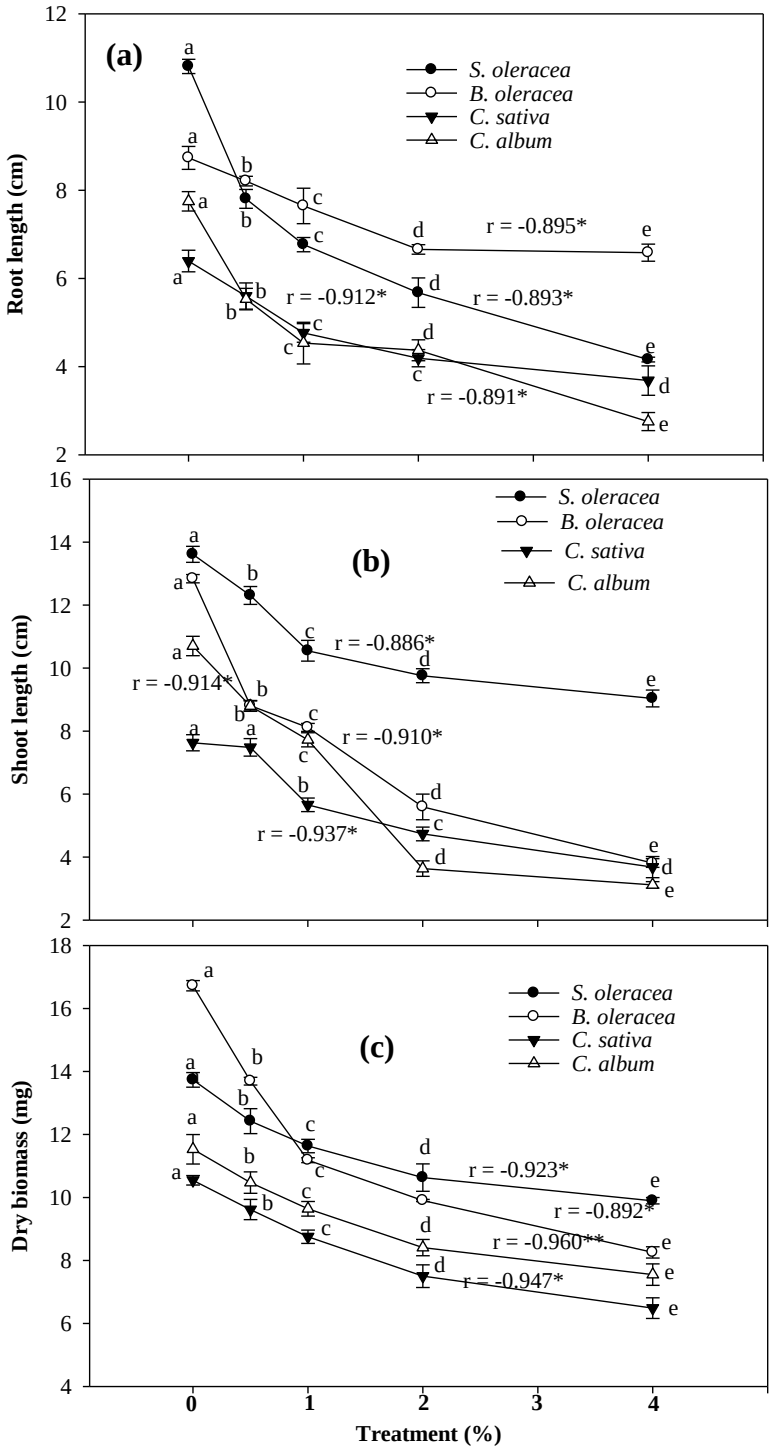
7.6.2. Shoot length

Similar with that of root length, the shoot length of *S. oleracea* was maximum (13.61±0.25 cm) followed by (12.84±0.13cm) in *B. oleracea* var. *botrytis,* (10.70±0.30 cm) in *C. album* and (7.63±0.25 cm) in *C. sativa in* control soil and it noticed that the shoot length reduced with increasing concentrations of root residue extract amended soil (Fig. 4.2b). The maximum reduction was noticed in *B. oleracea* var. *botrytis* (70.32%) and minimum in *S. oleracea* (33.65%). The correlation coefficient values (-0.886 to -0.914) between shoot length and concentration were strong and reciprocal representing a high degree of correlation between the two (Fig. 4.2b).

7.6.3. Dry biomass

The maximum dry biomass were seen in *B. oleracea* var. *botrytis* (16.72±0.16 mg/seedling) followed by (13.73±0.23 mg) in *S. oleracea,* (11.53±0.15 mg) in C *album* and (10.54±0.15 mg) in *C. sativa* in control set-up (Fig. 4.2c). Maximum retardatory effect in dry biomass was observed in *B. oleracea* var. *botrytis* (50.65%) followed by that of *C. sativa* (38.51%) with minimum in *S. oleracea* (27.96%) at the 4%. The correlation coefficient values between dry biomass and concentration were calculated to be strong and reciprocal with values ranging from -0.892 to -0.960.

Fig. 4.2: Allelopathic effect of root residue extract amended soil (RRES) on (a) root length (b) shoot length and (c) dry biomass of test plants (crops and weeds).



Different superscript symbols along a curve represent a significant difference among themselves at p<0.05 applying DMRT.
r represents correlation coefficient.
* and ** represent significance of correlation at p <0.05 and p<0.01, respectively.

7.7. Growth studies in RRE

7.7.1. Root Length

In control, the radicle length was measured to be maximum in *S. oleracea* (10.77±0.28 cm), *B. oleracea* var. *botrytis* (9.32±0.23 cm) and least in *C. sativa* (7.55±0.29 cm) followed by *C. album* (6.66±0.30 cm). But, when observed at 4% concentration, (40%) reduction in radicle length was seen in all test species, respectively. The growth was decreased with increasing concentration of aqueous extracts (Fig. 4.3a). In all the treatments given to test plants, a concentration based dose response relationship was observed. The correlation coefficient values between radicle length and concentration of all the test plants were calculated to be strong and reciprocal with values ranging from -0.929 to -0.983.

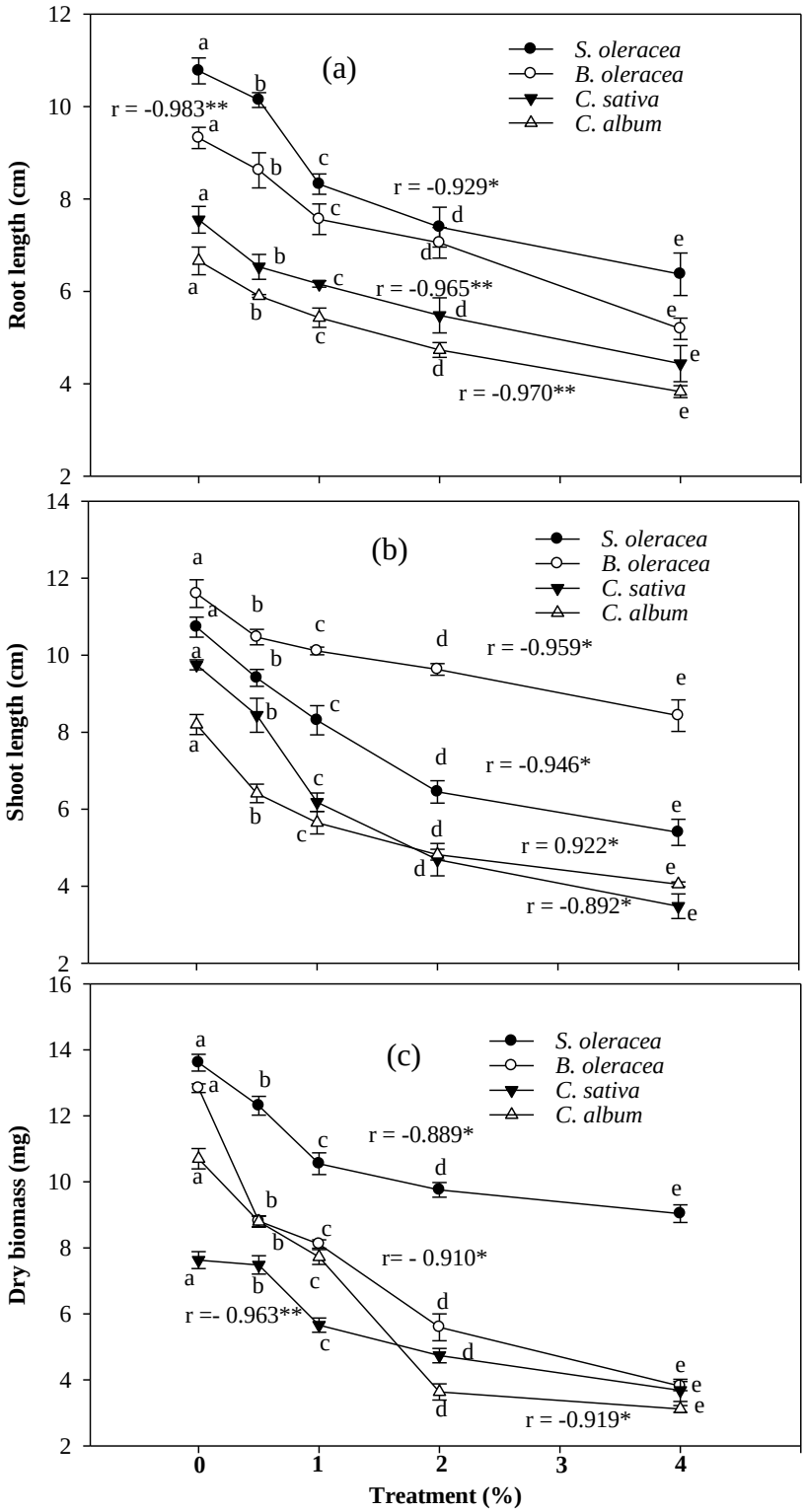
7.7.2. Shoot Length

Plumule length in control, was measured to be (10.73±0.26 cm) in *S. oleracea,* (11.60±0.36 cm) in *B. oleracea* var. *botrytis* and (9.75±0.13 cm) in *C. sativa,* respectively while the least (8.20±0.26 cm) was noticed in *C. album*. With increasing concentration of aqueous extracts of root residue, the reduction in plumule length was observed, which was statistically significant and appreciable (Fig. 4.3b) and least growth of the plumule was recorded at 4% concentration. Maximum effect on shoot length reduction was observed in case of *C. sativa* (64.30%) and minimum in case of *B. oleracea* var. *botrytis* reduced by (27.32%). The correlation coefficient between plumule length and concentration was calculated to be strong, reciprocal and ranging from -0.892 to -0.959.

7.7.3. Dry Biomass

Dry biomass was measured to be maximum in *S. oleracea* (13.61±0.25 mg/seedling) followed by *B. oleracea* var. *botrytis* (12.84±0.13 mg), *C. album* (10.70±0.30 mg) and *C. sativa* (7.63±0.25 mg), when treated with pure water (control). Similar to that of radicle and plumule length, the dry biomass of all the test plants were reduced with increasing concentration of aqueous extracts, which was statistically significant as compared to control (Fig. 4.3c). The maximum reduction of dry biomass was observed in *C. album* nearly (75.32%) and in *B. oleracea* var. *botrytis* (70.32%) at 4%. (Fig. 4.3c). A strong and reciprocal correlation coefficient between dry biomass and concentration with the values ranging from -0.889 to -0.963 was observed.

Fig. 4.3: Allelopathic effect of root residue extract (RRS) on (a) root length (b) shoot length and (c) dry biomass of test plants (crops and weeds).



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.

r represents correlation coefficient.

* and ** represent significance of correlation at $p < 0.05$ and $p<0.01$, respectively.

7.8. DYNAMIC OF RELEASE OF PHENOLIC CONTENT WITH TIME

7.8.1. Root residue soil (RES)

The amendment free (control) soil was estimated to contain (6.52±0.02 µg/g dry weight) of phenolic content. During the first hours (5-7), a gradual increase in the content of phenolics could be noticed. The maximum content (13.74±0.04 µg/g) was detected at 40 hours. After that, the phenolic content remained more or less constant at their respective hours with minimium content (13.37±0.01 µg/g) noticed at 120 hours of the amendment of residue (Fig 4.4b)

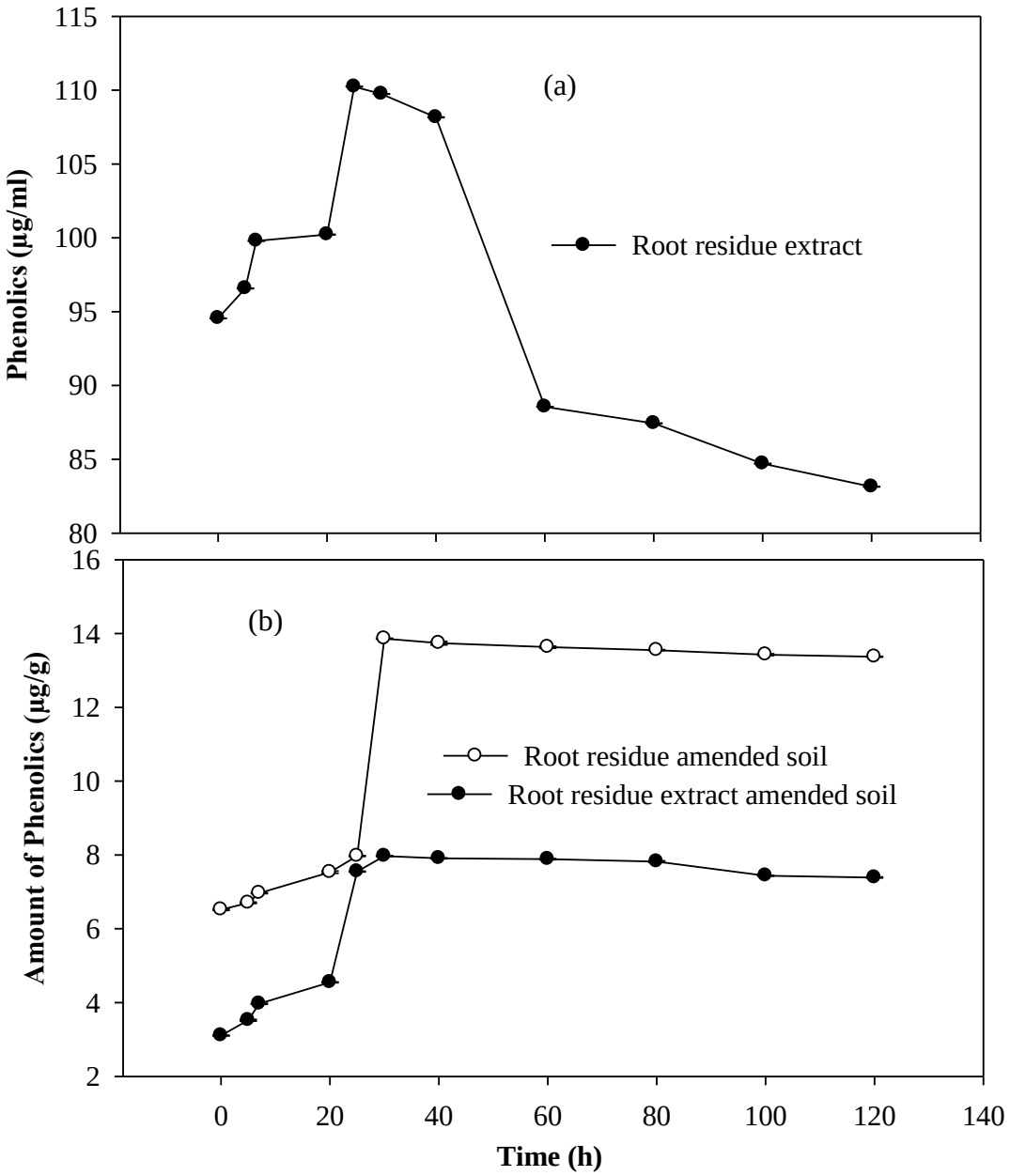
7.8.2. Root residue extract (RRE)

The phenolic content measured was (94.55±0.01 µg/ml) in amended free soil, but between 5 to 7 hours, phenolic content showed gradual increase (Fig. 4.4a) and the maximum amount (110.24±0.01 µg/ml) was noticed at 25 hours. Thereafter, it declined. The rate of decline was gradual especially till 40 hours of dipping. After 40 hours, a sharp decline in the content of phenolics was detected with minimum at 120 hours (Fig 4.4a).

7.8.3. Root residue extract soil (RRES)

The phenolic content detected in amended free soil was (3.11±0.01 µg/g dry weight). During the first hours (5-7), a gradual increase in the content of phenolics could be noticed. However, the maximum release of phenolics (15.69±0.09 µg/g) was detected at 40 hours. Afterwards, the release of phenolics declined almost constantly with minimum content (7.97±0.01 µg/g) reported at 130 hours. It is seen that of the two conditions, i.e. RRS and RRES showed difference in the content of free phenolics available in the medium (Fig. 4.4b).

Fig. 4.4: Dynamics of release of phenolics w.r.t. (a) root residue extract (b) root residue amended soil and root residue extract amended soil.



± represents standard deviation.

7.9. CHARACTERISTICS OF AQUEOUS EXTRACTS OF ROOT RESIDUE

7.9.1. pH

Aqueous extracts had a pH less than 7.0 irrespective of the concentration of root residue. With increasing concentration of extract, the pH value ranged from (5.28±0.20) to (6.54±0.04) (Table 4.2). The variations were statistically significant.

7.9.2. Electrical conductivity

The electrical conductivity of the root residue extract was relatively high. Its value increased from (0.89±0.05 mS) to (2.72±0.26 mS) with increasing concentration. The increase was over three times and showed a trend towards increase with increasing concentration (Table 4.2).

7.9.3. Phenolic content

The content of phenolics at 0.5% aqueous extract of root residue were calculated to be (101.89±3.84 µg/ml). With increasing concentration of aqueous extracts, the value increased sharply to the extent that at 4% concentration, it was measured to be (909.33±5.0 µg/ml) (Table 4.2).

Table 4.2: Estimation of pH, electrical conductivity and phenolic content in root residue extracts of *C. procera.*

Concentration (%)	pH	Electrical conductivity (µS)	Phenolic content (µg/ml)
0.5	6.54 ± 0.04^{a}	0.89 ± 0.05^{c}	101.89 ± 3.8^{c}
1.0	6.31 ± 0.18^{a}	1.04 ± 0.18^{c}	164.33 ± 4.3^{c}
2.0	5.88 ± 0.13^{b}	1.95 ± 0.07^{b}	431.00 ± 2.5^{b}
4.0	5.28 ± 0.20^{c}	2.72 ± 0.26^{a}	909.33 ± 5.0^{a}
LSD at 5%	**0.51**	**0.54**	**134**

Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.

± represents standard deviation.

7.10. ELEMENTAL ANALYSIS OF ROOT RESIDUE

When residue was analyzed for available elements, the plant was estimated to contain (0.19±0.02%) nitrogen, phosphorus (0.45±0.090%), potassium (1.86±0.10%) and sodium (0.58±0.10%) each on dry weight basis (Table 4.3). Among the macronutrients, the root residue constituted maximum amount of available calcium

with a value of (6.98±0.12%). Magnesium on the other hand, was estimated to be (2.46±0.49%). Among the micronutrients, iron showed maximum content with a value of (27.69±0.28 ppm), followed by Mn (3.72±0.20 ppm). The root residue was estimated to contain (1.96±0.16 ppm) and (0.76±0.17 ppm) of available Zn and Cu on the dry weight basis, respectively (Table 4.3).

Table 4.3: Elemental analysis of root residue of *C. procera.*

Element	Amount
Available N (%)	0.19^{ns}±0.025
Available P (%)	0.45±0.090
Available K (%)	1.86±0.10
Available Na (%)	0.58±0.10
Available Mg (g/100g)	2.46±0.49
Available Ca (g/100g)	6.98±0.12
Available Cu (ppm)	0.76±0.17
Available Zn (ppm)	1.96±0.16
Available Fe (ppm)	27.69±0.28
Available Mn (ppm)	3.72±0.20
LSD at 5%	**0.41**

±represent standard deviation; ns-nonsignificantly different

7.11. PHYSICO-CHEMICAL PROPERTIES OF AMENDED SOIL

7.11.1. pH

The amended free (control) soil showed a pH value of (7.48±0.05). The value increased with increasing concentration in both RRS and RRES. In case of RRS and RRES, this content relatively increased and it was measured to be (7.86±0.04) and (7.83±0.03) at 4% (Fig. 4.5a). The value of the correlation coefficient between pH and concentration of the amendment was calculated to be strong and positive (Fig. 4.5a).

7.11.2. Electrical conductivity

The electrical conductivity of unamended (control) soil was estimated to be (255.15±0.05 µS). After amendment of root residue or extract in the soil, an increase was observed, which was statistically significant at each concentration (Fig. 4.5a). In RRS and RRES, the electrical conductivity was estimated to be (489.56±0.071 µS)

and (290.53±0.07 µS), respectively at 4% (Fig. 4.5a). The values of correlation coefficients between electrical conductivity and concentration were calculated to be positive and strong.

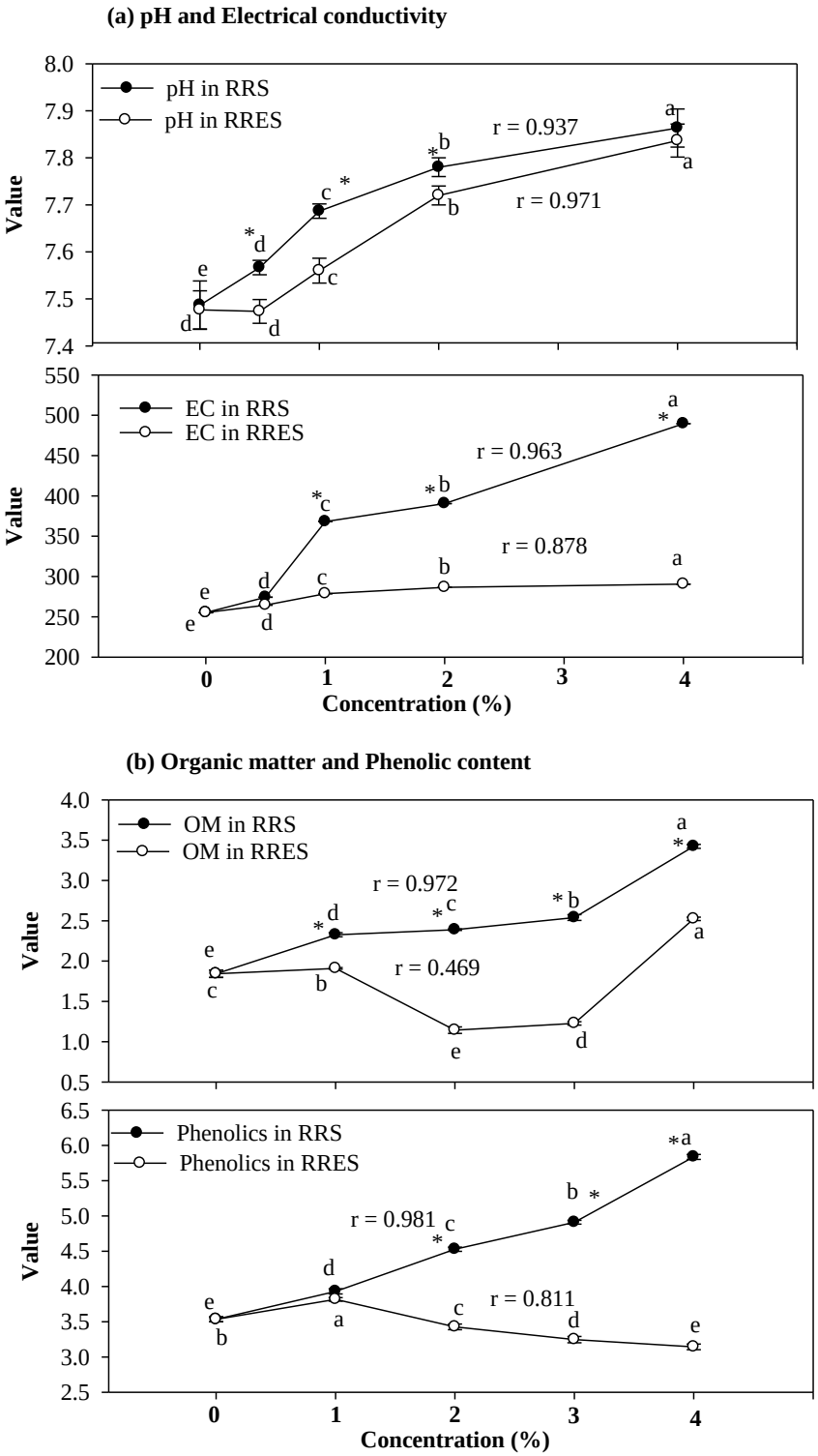
7.11.3. Phenolic content

The content of free phenolics in the control soils was estimated to be (3.53±0.035 mg/100g) dry weight. In contrast, in the amended soils (both RRS and RRES), it was more than that of control. It was seen to increase with increasing concentration of amendment (Fig. 4.5b). At the highest concentration of amendment, i.e. 4% of RRS, the amount of phenolic content increased two times and in case of RRES, the phenolic content showed a gradual decrease with increasing concentration respectively, than that of control. The differences were statistically significant except at 1% (Fig. 4.5b). The values of correlation coefficients (0.811-0.981) between phenolic content and amendment concentration were strong and positive.

7.11.4. Organic matter

The amendment free (control) soil was estimated to contain (1.84±0.04%) of organic matter. It increased with increasing the concentration of amended soil. However, the increase was not as sharp. In RRS and RRES, at highest concentration of amendment (4%), the values of organic matter were estimated to be (3.42±0.02%) and (2.52±0.02%), respectively (Fig. 4.5b). Further, the values of correlation coefficient were strong and positive.

Fig. 4.5 (a) pH and electrical conductivity (b) Phenolic content and organic matter in soil amended with different concentrations of root residue and root residue extract.



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

7.11.5. Available Nitrogen and Phosphorus

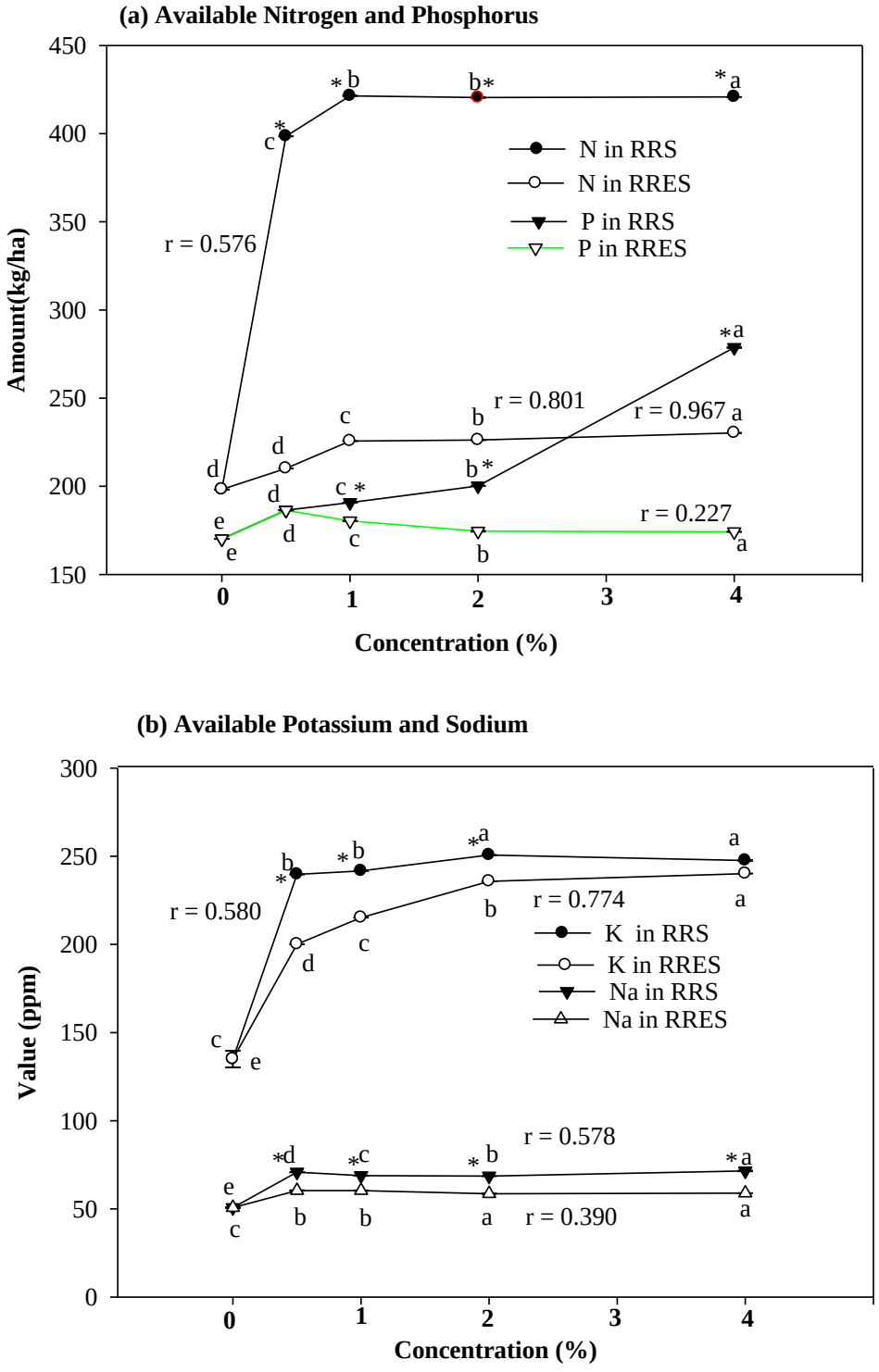
The unamended control soil was estimated to contain (198.18±0.06 kg/ha) of available nitrogen and (170.16±0.04 kg/ha) of available phosphorus. An increase in available nitrogen was observed in the amended soils and this increase was statistically significant at every concentration of RRS and RRES (Fig. 4.6a). In contrast to RRES, a sharp increase in nitrogen content was noticed in RRS. The difference in the amount of nitrogen at respective concentrations of the RRS and RRES was calculated to be again significant (Fig. 4.6a).

The amount of phosphorus was also more in the amended soils compared to unamended soil and showed an increase with increasing the concentration of amendments. Further, at higher concentrations the value of phosphorus was more in RRS compared to RRES. Between RRS and RRES, a statistically significant difference was observed at respective concentrations in both the elements except at 0.5%. The values of correlation coefficients between available nitrogen or phosphorus and concentration of amendment were calculated to be strong and positive.

7.11.6. Available Potassium and Sodium

The unamended (control) soil was estimated to contain (134.97±4.73 ppm) of potassium and (50.69±0.25 ppm) of sodium. The content of elements was relatively more in the amended soil compared to control. An increase was observed in both the elements in RRS and RRES (Fig. 4.6b). Between RRS and RRES, a statistically significant difference was observed at every respective concentration in both the elements. At the higher concentration (4%), the value of potassium was relatively more (247.57±0.32 ppm) in RRS compared to RRES (240.15±0.05 ppm) (Fig. 4.6b). A strong correlation coefficient between available elements and amendment concentration was also calculated.

Fig. 4.6: (a) Available Nitrogen and phosphorus (b) Potassium and sodium in soil amended with different concentrations of root residue and root residue extract.



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.
*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.
r represents correlation coefficient.

7.11.7. Available Calcium and Bicarbonates

In unamended control soil, the content of available bicarbonates was estimated to be (10.56±0.20 g/100g), while that of calcium (11.87±0.11 g/100) on dry weight basis. These ions were seen to be increased significantly in the amended soils. The content increased in the amended soils. This increase was constantly gradual and statistically significant at respective concentrations (Fig 4.7a).

In case of calcium, a statistically significant increase was observed with increasing concentration of amendment in both the cases (RRS and RRES). At the highest concentration of amendment (4%), the values were estimated to be (17.10±0.10 g/10g) and (16.32±0.06 g/10g) in RRS and RRES, respectively (Fig 4.7a). A very strong correlation coefficient between bicarbonate and amendment concentration was calculated. It was more than 0.760 in all the cases.

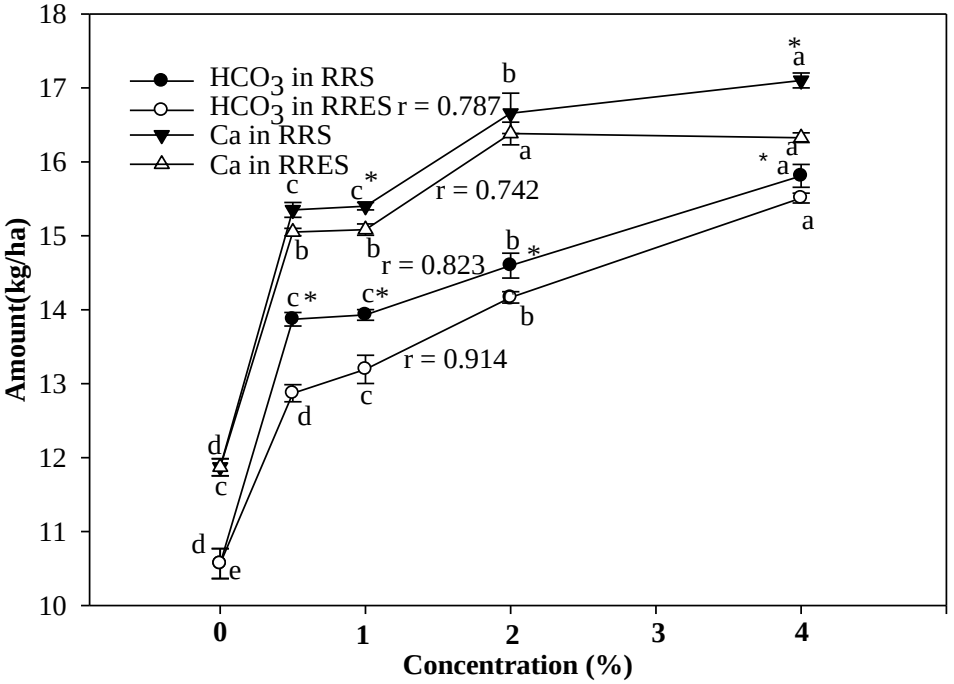
7.11.8. Available Chloride and Magnesium

The content of available magnesium was (3.25±0.07 g/100g) while that of chloride was (6.57±0.05 g/100g) of control soil. In chlorides, the linearity of increase was seen to be more in RRS rather than RRES (Fig. 4.7b). It was also apparent from the values of correlation coefficient, which was strong in RRS compared to RRES. Further, the variations in chloride between RRS and RRES at respective concentration were statistically significant (Fig 4.7b)

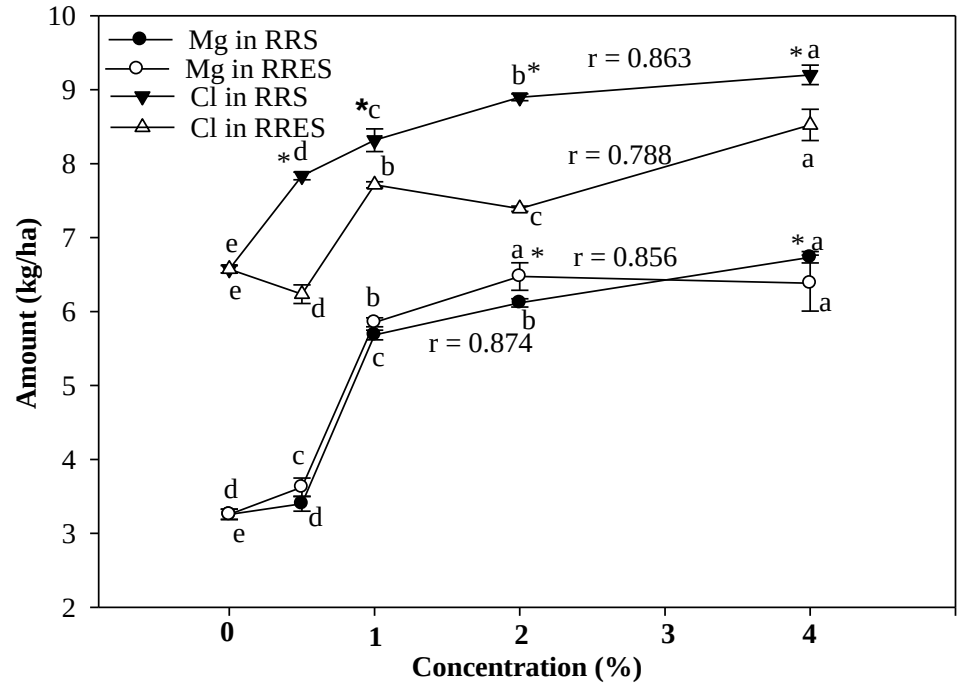
In case of Mg, at concentrations (0.5%, 1% and 2%), the amount was more in case of RRES and at higher concentrations (4%), the trend changed, it was more in RRS. In this case also, statistically significant variations between RRS and RRES were observed at higher concentrations, i.e. at 2% and 4% (Fig. 4.7b). Further, the values of the correlation coefficient in both the elements were high, i.e. more than 0.84, reflecting a strong relationship between each element and amendment concentration.

Fig. 4.7: (a) Available calcium and bicarbonates (b) Available magnesium and chlorine in soil amended with different concentrations of root residue and root residue extract.

(a) Available Bicarbonates and Calcium



(b) Available Magnesium and Chlorine



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

7.11.9. Available Micro-nutrients (Fe, Mn, Zn and Cu)

The unamended (control) soil was estimated to contain (14.77±0.23 ppm) Fe, (18.46±0.25 ppm) Mn, (3.48±0.07 ppm) Zn and (0.53±0.15 ppm) of Cu. All these micronutrients were increased in the amended soils.

In case of Fe, a statistically significant increase was observed in RRS and RRES with every increasing concentration and it was linear and consistent (Fig 4.8a). Further, it was also apparent from the values of correlation coefficient, which were positive and strong.

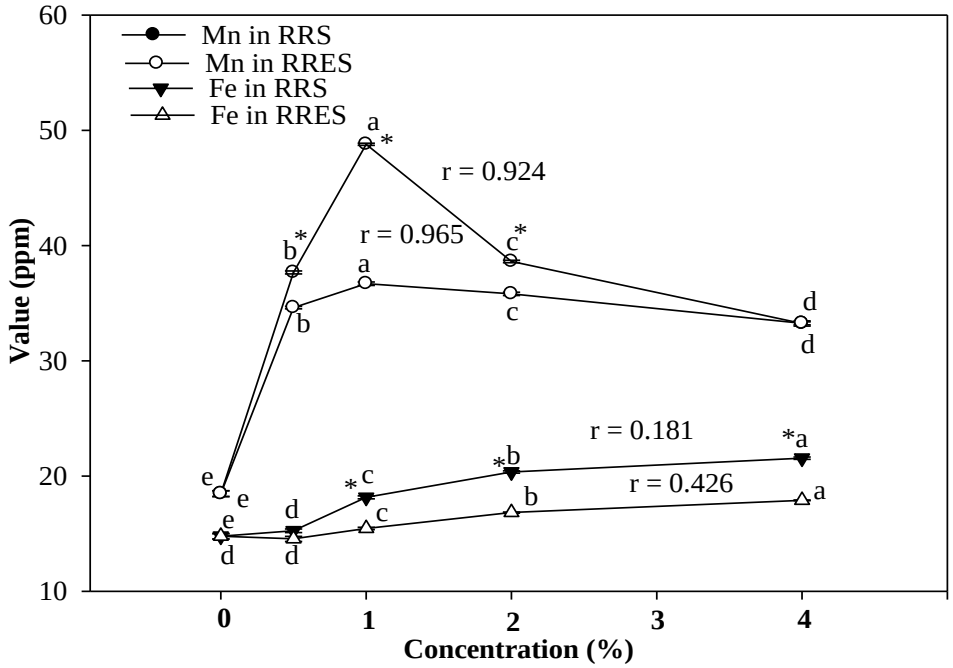
In case of Mn, the amount was increased at lower concentration (1% and 2%) of the amendment and after that, a sharp decline at 4% concentration in case of the RRS was noticed. A statistically significant difference between RRS and RRES was observed at lower concentrations (Fig 4.8a).

In case of Zn, a linear increase in values was not observed with increasing concentration of amendment (Fig 4.8b). However, statistically significant variations were observed at respective concentration, between RRS and RRES.

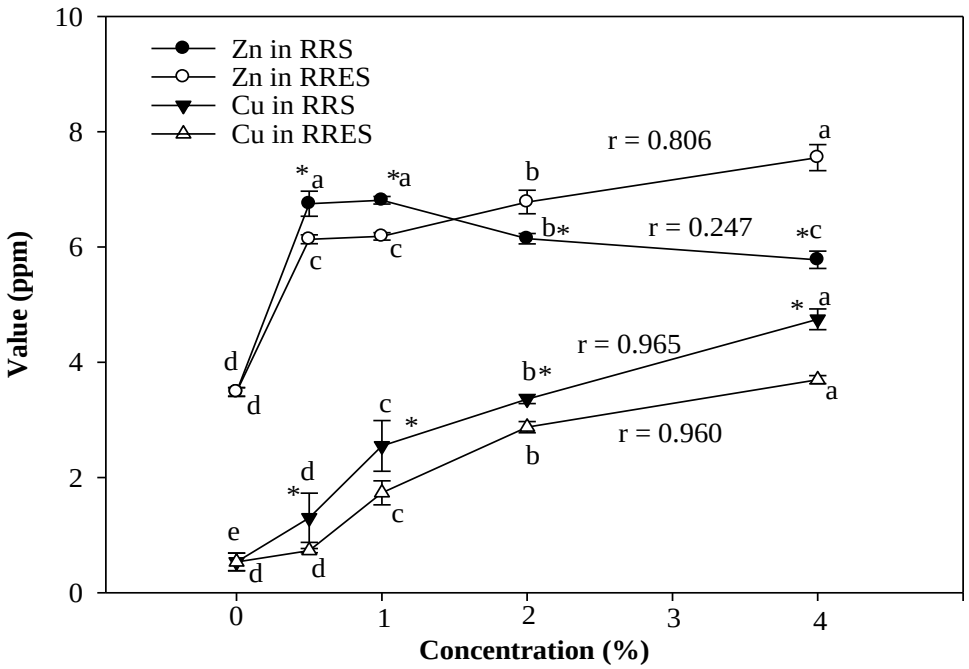
In case of Cu, with every increasing concentration, the content was increased. Statistically significant variations was observed in RRS and RRES at all concentration (Fig 4.8b). A strong correlation coefficient between concentration and content of Cu was reflecting the linear relationship.

Fig. 4.8 (a) Available Manganese and iron (b) Available zinc and copper in soil amended with different concentrations of root residue and root residue extract.

(a) Available Manganese and Iron



(b) Available Zinc and Copper



Different superscript symbols along a curve represent a significant difference among themselves at $p<0.05$ applying DMRT.

*represents significant difference between residue amended soil and residue extract amended soil at respective concentrations applying two sample t-test.

r represents correlation coefficient.

7.12. DISCUSSION

The roots have contributed to allelopathic interference has been revealed by several authors (Liu *et al*., 2005; Kong *et al*., 2006; Heidarzade *et al*., 2010; Hassan and Mathesius, 2012; Uddin *et al*., 2014). Anastomose branching of roots forms a jumbled biomass and are characterized by a well developed rhizosphere. Therefore, they are likely to contribute more towards the allelopathic interactions. Among these, roots being in direct contact with soil particles contribute phytotoxic metabolites into the surrounding environment (Bais *et al*., 2006; Batish *et al*., 2009b). In fact, allelochemicals release into the soil medium by a maximum production of root exudates (Bertin *et al*., 2003; Bais *et al*., 2004, 2006; Batish *et al*., 2007a) and plays a significant role in invasion process and regulating plant communities (Bais *et al*., 2004, 2006; Batish *et al*., 2007a; Batish *et al*., 2009b). A variety of compounds constitute root exudates such as amino acids, organic acids, sugars, phenolics and other secondary metabolites (Batish *et al*., 2007a) for regulation of interaction with other organisms through these constituents (bacteria, actinomycetes, pathogens, fungi, and insects) in the soil (Walker *et al.*, 2003b; Bais *et al.*, 2006). Contribution of roots and their exudates in allelopathic interactions needs to be explored essentially (Bais *et al*., 2006). Inhibitory effect in nature to a number of crops as *Lactuca sativa*, *Phaseolus vulgaris*, *Zea mays*, *Sorghum bicolor* noticed to be due to root exudates of *Bidens pilosa* (Stevens and Tang, 1985). Exudates were hydrophobic in nature that provided allelopathic properties to the weed (Friebe *et al.*, 1995; Singh *et al.*, 2001). From the plant root exudates, a number of phytotoxic compounds have been identified (Narwal *et al*., 2005). Indeed, the fate of allelopathic chemicals is determined by their retention, transformation and transport in soil and physicochemical and biological components of the soil and thus of allelopathy, in soil (Inderjit, 2001; Inderjit *et al*., 2010).

The present study is therefore, conducted with a view to understand the phytotoxic effect of its roots towards some crop and weed plants vis-à-vis interaction of their allelochemicals with soil properties. *C. procera* roots very deeply and rarely grow in soils that are shallow over unfractured rock with secondary and tertiary roots forming a network. During irrigation or by rainfall, release of allelochemicals occurs especially through leachation. In order to establish this, aqueous extract from the root residue of *C. procera* were prepared and *B. oleracea* var. *botrytis*, *S. oleracea*, *C.*

sativa and *C. album* were tested to their phytotoxicity. From the results, it is evident and already discussed, that aqueous extracts reduced the initial growth of test plants in terms of root length, shoot length and dry biomass. The maximum effect was observed on *C. album* and *C. sativa* and tested plants exhibited a differential activity towards the extracts. This clearly shows that aqueous extracts of *C. procera* roots possess growth inhibitors that are water soluble. Therefore, the phytotoxic nature of root residue extract gets further strengthened when these were incorporated into the soil. Similar results were reported by (Batish *et al.*, 2006a, 2007a; Batish *et al*., 2009b) due to negative effect of root exudates and residues of invasive weeds *Chenopodium album Chenopodium murale* and *Ageratium conyzoides*. Batish *et al*. (2009b) stated that root exudates and root residues of *A. conyzoides* impose an inhibitory effect satisfying its allelopathic behavior is in line with our study. In growth studies, soil amended with aqueous extract of the roots, induce the retardatory impact on growth of test plants (Javaid *et al.,* 2006; XiaoQing *et al.,* 2006). Further studies in view of the phytotoxic nature of roots and release of growth inhibitory substances, the soil was amended directly by the powdered root material. In this medium also, a significant reduction in test seedlings as compared to unamended control was observed.

In order to check the possible interactions of phenolics with soil nutrients and other soil properties, specific studies were also undertaken in this regard. Very less change in soil pH was observed. However, with the amendment of residues at 2% and 4% the soil was more alkaline i.e. pH increased towards alkalinity. In the present study, however, the analysis of the RRS and RRES indicated that availability of the nutrients is not a limiting factor and thus not a reason for the observed inhibitory effects is in agreement with the results of Batish *et al*. (2009b). The soils were nutrient rich, with an increased EC, indicating greater nutrient availability and thus enriching of the RRS and RRES. This is in line with the earlier reports where incorporation of residues or decomposing material of allelopathic plants into the soil can enhance nutrient status (Batish *et al*., 2002, 2007a; Batish *et al*., 2009b) and EC (Xuan *et al*., 2005; Batish *et al*., 2009b). Increase in the available soil nutrients, EC and pH of the soil have also been noticed upon amendation of decomposing residues of the invasive weed *Ageratium conyzoides* (Batish *et al*., 2009b), *Parthenium hysterophorus* (Batish *et al*., 2002) and C. *album* (Batish *et al*., 2006a). In the present

study, there was a substantial increase in the available N content as per study of Batish *et al*. (2009b). Moreover, phytotoxicity and quantification of phenolics from the RRS, RRES in pure form against test plants indicates their direct involvement in the observed growth reduction. Presence of significant amount of water soluble phenolics in the RRS and RRES indicated that these are primarily responsible for the observed growth reduction in these soils as has been previously investigated by Batish *et al*. (2009b). It is in agreement with earlier reports that from decomposing plant residues, including intact roots, water soluble phenolics are the ubiquitous organic biomolecules released and widely implicated in allelopathic interactions (Mizutani, 1999; Bertin *et al*., 2003: Xuan *et al*., 2005; Djurdjevic *et al*., 2008; Batish *et al*., 2009b). Batish *et al*. (2009b) reported that root residue amended soils possess phenolics and growth reduction has correlation with the content of phenolics. In particular, allelopathy of wild oat roots are mainly concerned with one of the main groups of substances known as phenolic compounds (Perez and Ormeno-Nunez, 1991). Seed germination and plant growth and other physiological processes get influenced by phenolic compounds (Djurdjevic *et al*., 2004). There may be a continuous flux of phenolics from the roots of *C. procera* into the immediate vicinity under natural field conditions, where they accumulate in bioactive concentration and thus interfere with other plant growth as revealed is in line with the study of Batish *et al*. (2009b).

Therefore, from the present study it can be concluded that release of allelochemicals by roots exerts allelopathic/phytotoxic effect on other plants. However, the ascertained contribution of roots in the overall influence of the *Calotropis* weed under natural conditions and they do play an important role in imparting allelopathic influence to the test plants. However, the level of contribution of the roots of the weed, especially, under natural conditions is not clearly evident. Otherwise, also, the variability in parameters in nature (soil texture, pH, humidity, nutrient contents and other competing vegetation) is so complex that generalization about their role in imparting allelopathic influence would not be advisable and convincing. We also hypothesize that roots (that remain hidden in the ground) of the weed exude some organic metabolites detrimental to the growth of associated crop plants which is in line with the study of Batish *et al*. (2009b). Further, Batish *et al*. (2009b) confirmed that before sowing of next crop, the farmers should remove the

aerial parts of the weed and the intact roots left within the soil may also contribute allelochemicals and thus interfere with the crop growth and yield.

Section V
Physiological Parameters

8. OBJECTIVE

The experiment was planned for the extraction of allelochemicals from the leaves of *Calotropis procera* (Ait.) R. Br. and to observe their impact on some crop and weed plants.

8.1. OBSERVATION PARAMETERS

1. Using solvent systems, allelochemicals (allelopathic chemicals) were extracted from the leaves of *C. procera*.
2. Treatment of test plants of *Triticum aestivum* L., *Spinacia oleracea* L., *Cannabis sativa* L. and *Chenopodium album* L. with aqueous and organic solvents, thus obtained.
3. Various growth parameters viz., carbohydrate content, protein content and chlorophyll content formed the parameters under study.

8.2. METHODOLOGY

Fresh**,** healthy leaves of *C. procera* were collected from dense population of weed from the University campus. These were grouped into two halves. The method described in a Protocol-I (Chapter 3, Materials and Methods) was followed for the recovery of aqueous leachates in the first half and the second half were shade dried and powdered. Various polar and non-polar solvent systems given in Protocol II (Chapter 3, Materials and Methods) were employed for organic extract fractionation from powder. From cultivated fields as well as weed thickets, the test plants were selected, uprooted from the soil and replanted in earthen pots of 4 cm diameter. The plants were thinned to six and for each treatment, five replicates were maintained.

Treatment solutions extracted from the leaves of *C. procera* constitutes aqueous leachates (1% g/ml) fresh wt. in pure water, petroleum ether fraction (0.9% w/v), methanolic fraction (0.9% w/v), chloroform fraction (0.9% w/v) and water fraction (0.9% w/v). The comparable treatment with pure water served as control in all the treatments. The concentrations of various fractions under experimentation were decided on the basis of a pilot experiment on the % of germination where a wide range was used. The one that showed 50±10 percent values served as LC_{50} (Lethal concentration killing 50% of the population).

8.2.1. Treatment to mature plants

For the estimation of various macromolecular content (carbohydrate content, protein content and chlorophyll content), six plants for each treatment were sprayed

with 100 ml of the treatment solution per plant daily for five days (Gulzar and Siddiqui, 2014b). On the sixth day, the estimation of the carbohydrate, protein and chlorophyll content were made from the freshly plucked leaves following procedures given in the (Chapter 3, Materials and Methods).

8.3. STATISTICAL ANALYSIS

The whole experiment was repeated. To assess the significance of differences data of mean values was analyzed by ANOVA (Analysis of variance) between various treatments. Data has also been represented graphically in the form of bar diagrams.

8.4. RESULTS

8.4.1. Carbohydrate content

In case of *T. aestivum*, the acid soluble and water-soluble carbohydrates content were (50.63±0.45 mg/g dry wt.) and (58.65±0.46 mg/g dry wt.), respectively in samples treated with pure water. However, the carbohydrate content showed a remarkable increase of (acid solubleand water soluble) as compared to those plants, which were treated, with water as a control (Fig. 5.1, 5.2). Maximum amount was noticed in samples treated with aqueous leachates as compared to the other treatments. All the other treatments of the organic extract fractions also had increased carbohydrate content, but it was much less, than that of aqueous leachate treated sample. Further, the differences were statistically significant (Table 5.1). Except water fraction treated sample, water soluble carbohydrate of *T. aestivum* was more than the acid soluble carbohydrates, but they too showed a similar trend of increase as the acid soluble carbohydrates (Table 5.1).

S. oleracea also had a marked effect of treatment solutions, but it was less pronounced as compared to *T. aestivum.* In control treated sample, the acid soluble carbohydrates were (32.21±0.38 mg/g dry wt.). Like *T. aestivum*, as compared to the other treatments, the aqueous leachates treated samples had a very high content of carbohydrates (Fig. 5.1). However, the amount of carbohydrate content (46.72±0.45 mg/g dry wt.) was least affected by the water fraction treatment (Table 5.1). The water soluble carbohydrate of *S. oleraceaa* in control noticed was (38.26±0.54 mg/g dry wt.) and were under maximum stress from the aqueous leachates treated sample (Fig. 5.2) Almost same effects were observed in petroleum and the methanolic

fraction treated sample. However, least amount of carbohydrate was detected in water fraction treated sample.

The acid soluble carbohydrate content of *C. album* was (54.40±0.52 mg/g dry wt.) in the control, while the content was maximum (84.91±0.13 mg/g dry wt.) of the aqueous leachates treated sample (Table 5.1). As compared to the other treatment fraction, the methanolic fraction treated samples had a lesser content (49.98±0.13), though the values were more than the control (Fig. 5.1). The highest content of water soluble carbohydrate (96.68±0.56 mg/g dry wt.) of *C. album* was noticed in aqueous leachates treated sample. In the petroleum ether and chloroform fraction treated sample, almost same amount of carbohydrate with the value of (82.67±0.29 mg/g dry wt.) and (82.72±0.35 mg/g dry wt.), respectively was calculated (Fig. 5.2). Minor difference was observed for carbohydrate content between the aqueous leachate and water fraction treated sample, it reduced by 4.14% in water fraction compared to the aqueous leachates (Table 5.1).

The acid soluble carbohydrate content of *C. sativa* in the control was (49.16±0.17 mg/g dry wt.). Aqueous leachates and petroleum ether treated samples had 101.55% and 67.02% more amount as compared to control (Fig. 5.1). Methanolic fraction and water fraction exhibited a gradual decrease in their activity. In both cases (acid soluble and water soluble), the amounts of carbohydrates were more than the control (Table 5.1). The differences were statistically significant.

Table 5.1: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on total carbohydrate content of *T. aestivum*, *S. oleracea*, *C. album* and *C. sativa.*

Treatment Solutions	*T. aestivum*		*S. oleracea*		*C. album*		*C. sativa*	
	Acid soluble carbohydrates (mg/g dry wt.)	Water soluble carbohydrates (mg/g dry wt.)	Acid soluble carbohydrates (mg/g dry wt.)	Water soluble carbohydrates (mg/g dry wt.)	Acid soluble carbohydrates (mg/g dry wt.)	Water soluble carbohydrates (mg/g dry wt.)	Acid soluble carbohydrates (mg/g dry wt.)	Water soluble carbohydrates (mg/g dry wt.)
Control	50.63±0.45^{f}	58.65±0.46^{f}	32.21±0.38^{f}	38.26±0.54^{e}	54.40±0.52^{d}	53.18±0.31^{e}	49.16±0.17^{f}	61.14±0.29^{e}
Aqueous leachates AL (1% g/ml fresh wt.)	89.85±0.48^{a}	92.66±0.44^{a}	85.51±0.42^{a}	81.74±0.39^{a}	84.91±0.13^{a}	96.68±0.56^{a}	99.08±0.71^{a}	97.71±0.34^{a}
Petroleum ether Fraction PF (0.09% w/v)	72.74±0.46^{c}	74.86±0.66^{c}	71.74±0.37^{c}	73.91±0.11b^{c}	66.16±0.51^{c}	82.67±0.29^{c}	82.11±0.39^{b}	85.14±0.66^{b}
Methanolic fraction MF (0.09% w/v)	53.92±0.45^{e}	70.94±0.72^{d}	75.00±0.11^{b}	73.70±0.32^{c}	49.98±0.13^{d}	60.96±0.06^{d}	64.58±0.64^{d}	85.22±0.71^{b}
Chloroform fraction CF (0.09% w/v)	79.80±0.42^{b}	82.96±0.16^{b}	70.14±0.19^{d}	74.62±0.18^{b}	74.78±0.46^{b}	82.72±0.35^{c}	53.55±0.50^{e}	82.80±0.30^{c}
Water fracrtion WF (0.09% w/v)	67.94±0.55^{d}	61.86±0.55^{e}	46.72±0.45^{e}	51.37±0.73^{d}	66.70±0.32^{c}	92.67±0.47^{b}	69.72±0.47^{c}	73.79±0.32^{d}
LSD at 5%	**1.06**	**1.43**	**0.87**	**1.10**	**0.98**	**0.95**	**1.30**	**1.18**

Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.

± represents standard deviation.

8.4.2. Protein content

In case of *T. aestivum,* the protein content was (61.13±0.14 mg/g dry wt.) in control and it was decreased in all treatments compared to control (Table 5.2, Fig. 5.3). However, in the water fraction treated sample, maximum decrease was calculated (34.71±0.47 mg/g dry wt.) with a reduction of (43.21%) followed by chloroform fraction treatment (Fig. 5.3).

In case of *S. oleracea*, the protein content of (52.26±0.17 mg/g dry wt.) was observed in control (Table 5.2). In all these treatments, the decrease was statistically significant compared to control. Maximum reduction (31.51±0.37, 39.71%) was observed in the water fraction treated sample. However, petroleum ether fraction (35.63±0.55 mg/g dry wt., 31.83%) and methanolic fraction (35.32±0.40 mg/g dry wt., 32.42%) exhibited almost similar difference in protein content reduction compared to control (Fig. 5.3)

Progressive decrease in the total protein content of *C. album* was observed in all the treatment solutions. This decrease with respect to control (65.55±0.37 mg/g dry wt.) was highly significant statistically (Table 5.2). The protein contents were decreased by (19.70%) and (25.88%) in case of aqueous leachates and petroleum ether fraction treated sample, respectively. In methanolic fraction treated sample maximum decrease about (37.05%) and in chloroform fraction only (15.54%) reduction in protein content was calculated compared to control (Fig. 5.3).

A similar observation was also detected in *C. sativa* as in *T. aestivum, S. oleracea* and *C. album*. Compared to control setup, protein content was decreased in all treatments (Fig 5.3). Maximum reduction was observed in the water fraction treated sample (34.86%) followed by chloroform (31.98%) and methanolic fraction (29.13%). Almost similar reduction, (15.52%) and (14.53%) in the aqueous leachates fraction and petroleum ether fraction, respectively was detected (Table 5.2).

Table 5.2: Impact of aqueous leachates and organic solvents extracted from the *C. procera* leaves on total protein content of *T. aestivum*, *S. oleracea*, *C. album* and *C. sativa*.

Treatment solutions	Total protein content (mg/g dry wt.)			
	T. aestivum	***S. oleracea***	***C. album***	***C. sativa***
Control	61.13±0.14^{a}	52.26±0.17^{a}	65.55±0.37^{a}	70.77±1.45^{a}
Aqueous leachates AL (1% g/ml fresh wt.)	38.34±0.17^{e}	42.49±0.35^{b}	52.64±0.55^{c}	59.79±0.58^{b}
Petroleum ether fraction PF (0.09% w/v)	40.78±0.60^{d}	35.63±0.55^{c}	48.58±0.88^{d}	60.49±1.49^{b}
Methanolic fraction MF (0.09% w/v)	51.80±1.42^{b}	35.32±0.40^{c}	41.27±0.59^{f}	50.16±0.99^{c}
Chloroform fraction CF (0.09% w/v)	47.07±0.25^{c}	43.14±0.37^{b}	55.36±0.52^{b}	48.14±0.90^{d}
Water fracrtion WF (0.09% w/v)	34.71±0.47^{f}	31.51±0.37^{d}	44.59±0.66^{e}	46.10±0.15^{e}
LSD at 5%	**1.86**	**0.98**	**1.57**	**2.64**

Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

8.4.3. Chlorophyll content

In case of *T. aestivum*, the total chlorophyll content (7.99±0.86 µg/mg fresh wt.) was observed in control and it was decreased in aqueous leachates and organic fractions treated sample (Fig 5.4, Table 5.3). The maximum and minimum reduction in chlorophyll content was calculated (68.47%) in chloroform fraction and (27.29%) in aqueous leachates, respectively (Table 5.3).

In *S. oleracea*, the chlorophyll content was (5.59±0.52 µg/mg fresh wt.) in control and in methanolic fraction treated sample maximum reduction was observed, which was (40.61%) and followed by (26.48%) in a chloroform fraction (Table 5.3). In all treatment, the reduction was statistically significant (Fig 5.4).

Table 5.3: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on total chlorophyll content of *T. aestivum*, *S. oleracea*, *C. album* and *C. sativa*.

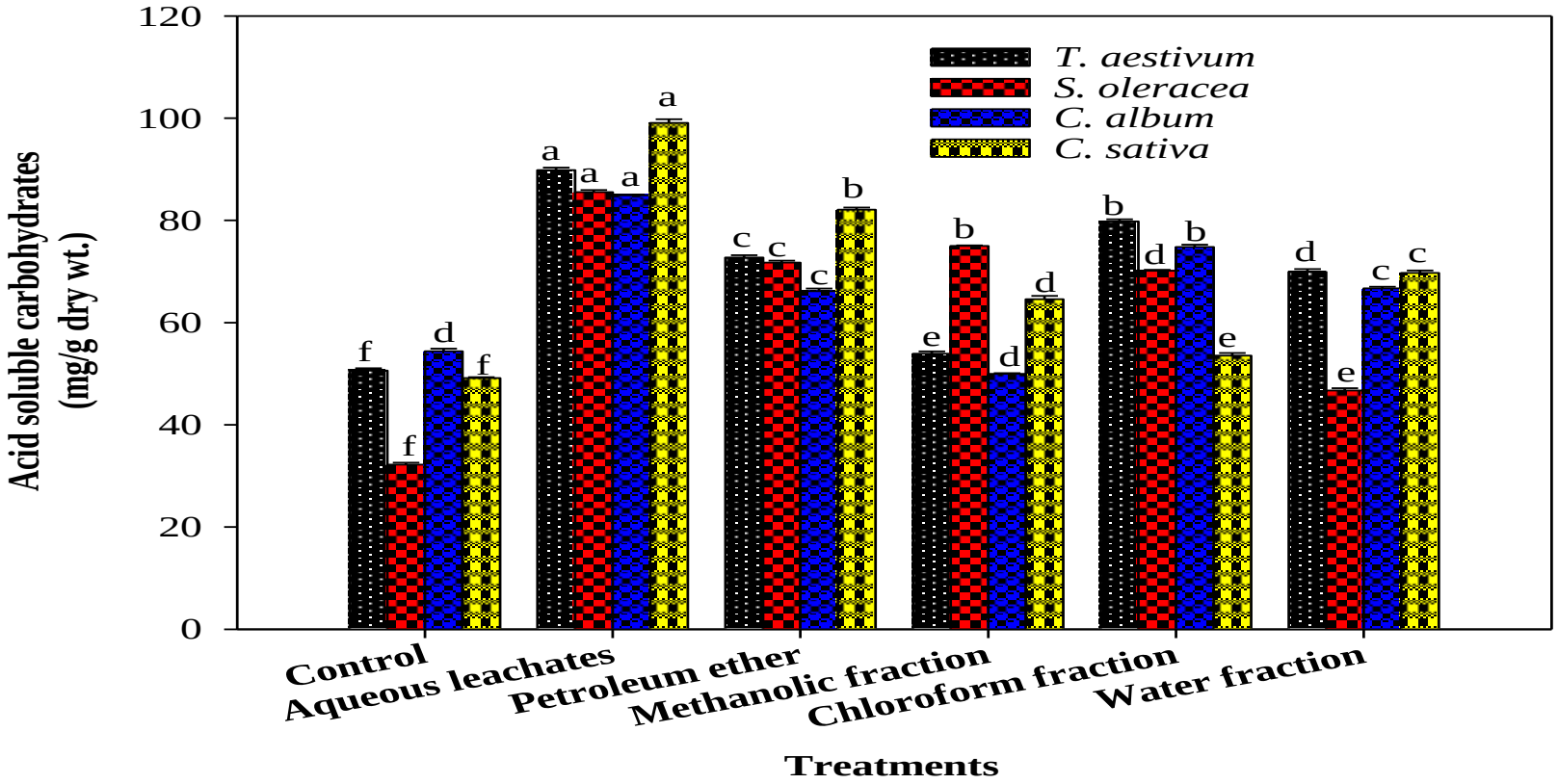
Treatment solutions	Total chlorophyll content (µg/mg fresh wt.)			
	T. aestivum	***S. oleracea***	***C. album***	***C. sativa***
Control	7.99 ± 0.86^{a}	5.59 ± 0.52^{a}	4.73 ± 0.28^{a}	6.83 ± 0.42^{a}
Aqueous leachates AL (1% g/ml fresh wt.)	5.81 ± 0.92^{b}	4.11 ± 0.21^{b}	3.16 ± 0.18^{bc}	4.33 ± 0.20^{b}
Petroleum ether Fraction PF (0.09% w/v)	3.55 ± 0.50^{bc}	5.25 ± 0.58^{a}	3.40 ± 0.46^{b}	3.58 ± 0.26^{c}
Methanolic fraction MF (0.09% w/v)	4.32 ± 0.42^{c}	3.32 ± 0.39^{c}	2.58 ± 0.65^{c}	3.79 ± 0.13^{c}
Chloroform fraction CF (0.09% w/v)	2.52 ± 0.45^{d}	3.87 ± 0.36^{bc}	3.26 ± 0.49^{bc}	6.48 ± 0.29^{a}
Water fracrtion WF (0.09% w/v)	3.93 ± 0.07^{c}	4.25 ± 0.28^{b}	1.17 ± 0.12^{d}	4.54 ± 0.34^{b}
LSD at 5%	**1.52**	**1.04**	**1.04**	**0.61**

Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

In *C. album*, the total chlorophyll content was (4.73±0.28 µg/mg fresh wt.) in the control while it was decreased in the water fraction by (75.27%) followed by a methanolic fraction (45.46%), aqueous leachate fraction (33.20%), chloroform fraction (31.08%) and in petroleum ether fraction treated samples it was only (28.12%) (Table 5.3). The petroleum ether fraction had no much difference in the amount of content of chlorophyll (Fig. 5.4).

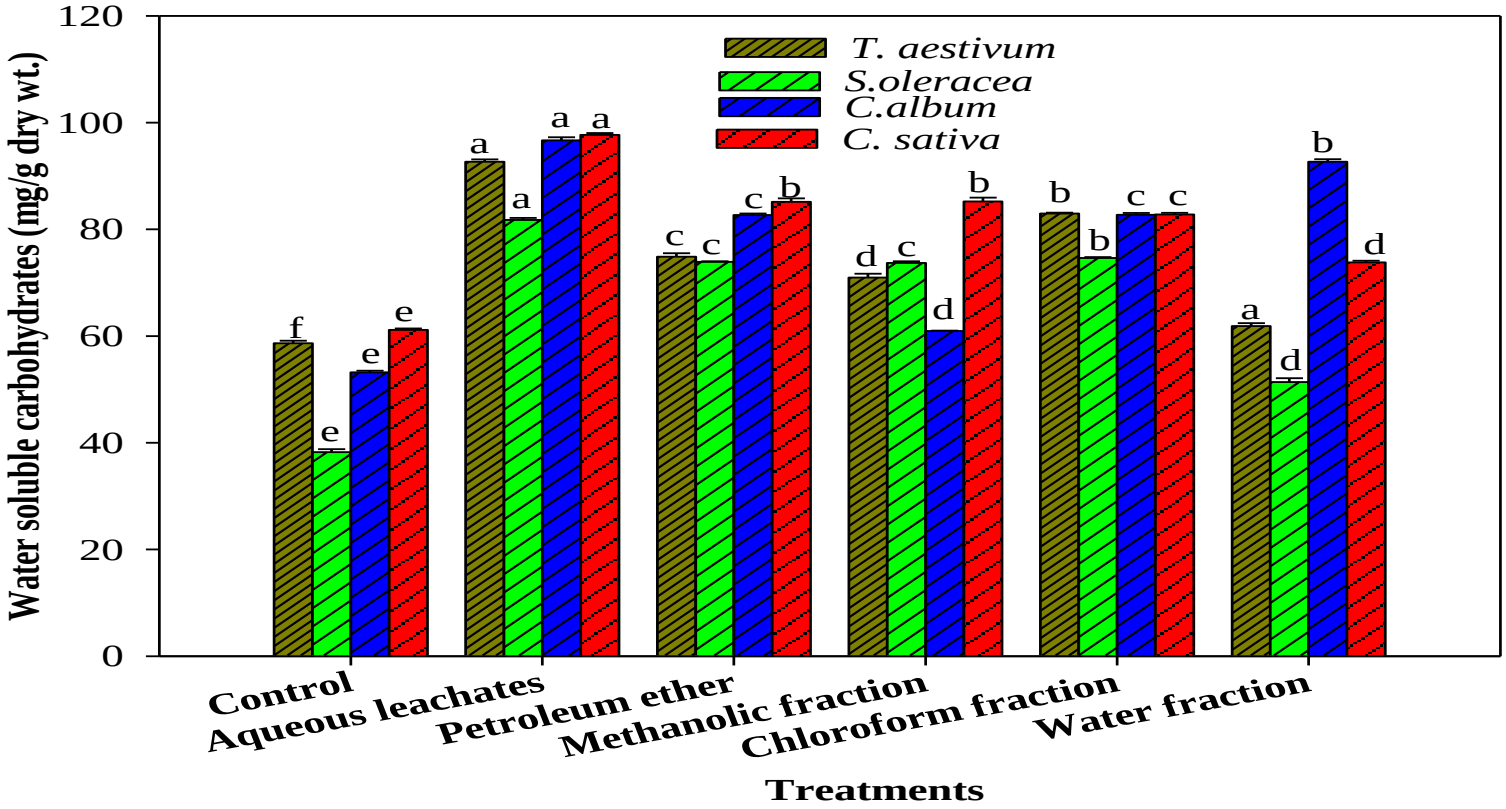
The chlorophyll content of the control set was (6.83±0.51 µg/mg fresh wt.) in *C. sativa*. The petroleum ether fraction, methanolic fraction and aqueous leachates exhibited the decrease in chlorophyll content in terms of (50.81%), (45.37%) and (39.98%), respectively (Table 5.3). However, the value (4.54±0.34 µg/mg fresh wt.) kept increasing from the treatment of water fraction and it further increased in a chloroform fraction (6.48±0.29 µg/mg fresh wt.) but was less than control. The petroleum ether fraction and methanolic fraction had the worst effect on the amount of chlorophyll by decreasing it to almost half at 3.36±0.39 µg/mg fresh. wt. and 3.73± 0.64 µg/mg fresh. wt., respectively (Fig. 5.4).

Fig. 5.1: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on acid soluble carbohydrate content of test species.



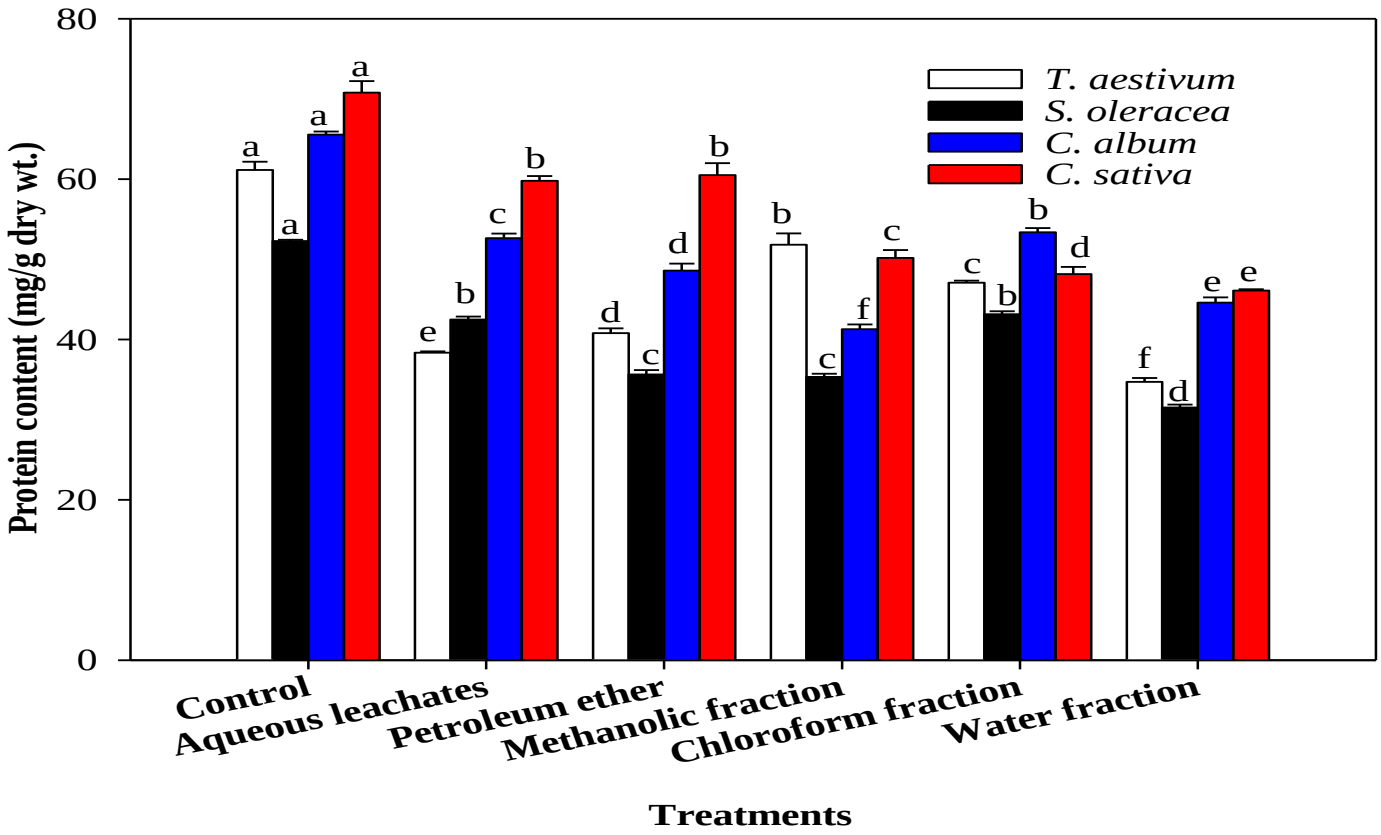
Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

Fig. 5.2: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on water soluble carbohydrate content of test species.



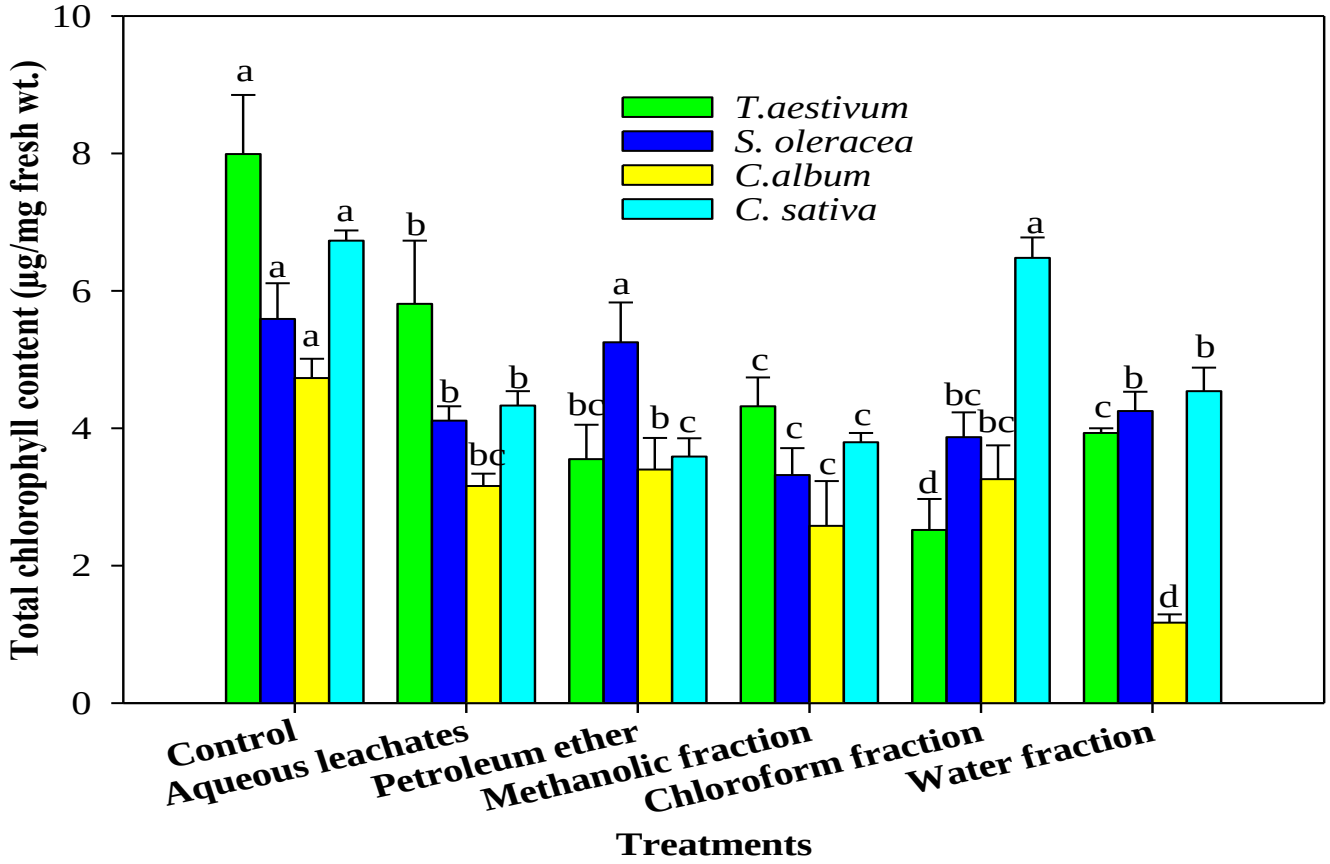
Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

Fig. 5.3: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on total protein content of test species.



Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

Fig. 5.4: Impact of aqueous leachates and organic solvents extracted from *C. procera* leaves on total chlorophyll content of test species.



Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

8.5. DISCUSSION

Allelochemicals interfere with the growth, development and performance of a plant through their effect directly or indirectly on metabolism (Raoof and Siddiqui, 2013a). Since we are trying to find out the effect of allelochemicals through three major metabolic processes, i.e. carbohydrate synthesis, protein synthesis and photosynthesis, hence they have discussed in the experiments. Many physiological parameters get altered by the allelopathic impact of allelochemicals. They mainly include cell division, cell differentiation, ion and water uptake, water status, phytohormone metabolism, respiration, photosynthesis, enzyme function, signal transduction as well as gene expression, transpiration, water utilization, reactive oxygen species generation, photosystem II (PSII) efficiency, dark respiration, ATP synthesis and cell cycle (Reigosa *et al*., 1999a; Inderjit and Duke, 2003; Hejl and Koster, 2004; Zhou and Yu, 2006; Field *et al*., 2006; Ding *et al*., 2007; Lorenzo *et al*., 2008; Chobot *et al*., 2009; El-Khatib *et al*., 2016).

As compared to the control, chlorophyll content in both crops and weeds decreased dramatically and the aqueous leachates proved to be much more effective than the other treatment (Gulzar and Siddiqui, 2014b) in this case too. There are several reports that the chlorophyll content of leaves decreased under stressful conditions (Ibrahim *et al*., 2013). Several studies reported the decrease in chlorophyll contents with increase in concentration of allelopathic phenolics (vanillic acid, *o*-hydroxyphenyl acetic, *p*-hydroxybenzoic acid, ferulic and *p*-coumaric acids) in rice cabbage, Chinese fir, *Echinochloa crus-galli*, *Chenopodium album*, maize (Chen *et al*., 2002; Yang *et al*., 2004; Al-Sobhi *et al*., 2006; Jaleel *et al*., 2008; Sarkar *et al*., 2012; Singh *et al*., 2013; Namkeleja *et al*., 2014) that coincides with our results. Either the chlorophyll synthesis gets inhibited or excessive breakdown of chlorophyll by acting on the pyrrolic ring and the phytol chain or decreased dry matter, i.e. photosynthates also results due to inhibition of photosynthesis under the influence of allelochemicals (Rice, 1984; Colton and Einhelling, 1980; Blum *et al*., 1985; Ervin and Wetzel, 2000; Moradshahi *et al.,* 2003; Bajaj *et al.,* 2004; Thapar and Singh, 2006; Yang *et al.,* 2006; Singh *et al*., 2009; Ibrahim *et al*., 2013). As stated by (Kapoor, 2012), allelochemicals present in the leaf leachate/extract might result in a reduction of chlorophyll content by interacting with phosphorylation pathway or inhibition in activation of Mg^{2+} and ATPase activity or other metabolic activities.

Colton and Einhellig (1980) reported that the allelochemicals inhibit the rate of photosynthesis due to interference with water balance and chlorophyll content. The performance of three main processes of photosynthesis (stomatal control of carbon dioxide supply, thylakoid electron transport and the carbon reduction cycle) gets affected by allelochemicals (Einhellig *et al*., 1993; Namkeleja *et al*., 2014). For photosynthesis, chlorophyll is the most important pigment (Niinemets and Tenhunen, 1997). For the conversion of light energy (solar radiation) to stored chemical energy, the chlorophyll a and b are essential pigments (Gitelson *et al.*, 2003; Namkeleja *et al*., 2014). Chlorophyll contents determine the photosynthetic potential of a plant (Li *et al.*, 2011b) and change in photosynthesis is expected to bring any changes (Curran *et al*., 1990). Because much of leaf nitrogen is incorporated in chlorophyll, therefore chlorophyll also gives an estimation of the plant nutrient status (Filella *et al*., 1995; Namkeleja *et al*., 2014). The overall plant healthy condition is also influenced by the amount of chlorophyll per unit leaf area in plant (Filella *et al*., 1995; Namkeleja *et al*., 2014). Larger amounts of chlorophyll reveal the maximum growth of healthy plants than unhealthy ones (Wu *et al*., 2008). Hence, the photosynthesis gets inhibited by allelochemicals which ultimately can lead to the death of the plant.

One thing is very clear from the result of this experiment that *C. procera* leaves allelopathic exert a very negative influence on the acid soluble and water soluble carbohydrates (Gulzar and Siddiqui, 2014b) of test species. It is very well depicted by an increased amount of carbohydrate content exerts its influence mainly through its aqueous leachates, i.e. in its glucosidic form (Gulzar and Siddiqui, 2014b). The weed plants show more sensitivity as compared to crop plants though, it did get affected by the allelochemicals (Colton and Einhelling, 1980; Blum *et al*., 1985). The results are in line with findings of (Sahar *et al*., 2005; Abdulghader *et al*., 2008; Gulzar and Siddiqui, 2014b; Ibrahim *et al*., 2013) where an appreciable increase in the increased concentration of soluble sugars occurred in response to various allelopathic agents. Synthesis of carbohydrates takes place in the living tissue. An increased amount of carbohydrates points out to the fact that the plant is under stress and it is gathering up its energy reserves to meet any conditions of adversity. Recently, there been increased research on the role of the demand for photoassimilates in regulating photosynthesis through changes in carbohydrate partitioning and accumulation under stress conditions (Levitt, 1982; Osmond *et al*.,

1987; Paul and Driscoll, 1997; Paul and Foyer, 2001; Pieters and Souki, 2005). In response to the variety of environmental stresses, accumulation of sugars in different parts of plants is enhanced (Prado *et al*., 2000; Khan and Naqvi, 2012).

It was observed that the plant protein content was found to be reduced in all the treatments as compared to control. The findings in the present investigation is in line with these reports (Iman *et al*., 2006; Abu-Romman *et al*., 2012; Ibrahim *et al*., 2013, Gulzar and Siddiqui, 2014b) with decreased amount of protein. Reduction in the rate of protein synthesis occurred by incorporation of certain amino acids into proteins (Baziramakenga *et al*., 1997). Besides, the activities of many enzymes are hampered by the phenolic acids have been shown by Hopkins (1999). Highest application of allelochemicals decreased the protein content and this may be due to the accumulation of phenolic glycine that interferes with the cytoplasmic ribosomes and production of RNA, which in turn inhibited protein synthesis (Hegab and Ghareib, 2010). Increased amount of free amino acids has been observed upon protein degradation due to the allelochemicals (Singh and Thapar, 2003). The reduction of protein content under various treatments could be attributed to impairment of various metabolic activities by leachates, which inhibit the protein synthesis and/or stimulate the degradation as suggested by Mersie and Singh (1993) or it could be due to protease activity as reported by Singh *et al*. (2002). Allelochemicals inhibit the rate of photosynthesis due to interference with water balance and chlorophyll contents which may result in a reduction in the amount of protein.

It is believed, different allelochemicals or herbicides exert variety of mechanisms of action. Nevertheless, it is difficult to determine the primary mechanism involved for the actions of these chemicals (Einhellig, 2009). Even a specific compound may affect several metabolic functions and as a result, it is seldom possible to sort out primary effects from the secondary ones (Einhellig, 2009). In addition, the uncertainty in interpreting the observed effects in isolated enzymes to other biochemical effects in intact plant system also exists. No doubt, allelochemicals or herbicides (natural or synthetic) act on plants though enzymatically controlled reactions. To solve this mystery research is needed at the molecular level (Gulzar and Siddiqui, 2014b).

Section VI

Germination Parameters

9. OBJECTIVE

Extraction of allelochemicals from leaves of *Calotropis procera (*Ait.) R. Br. and to investigate their bioefficacy on some weed and crop plants was planned.

9.1. OBSERVATION PARAMETERS

1. Extraction of allelochemicals (the allelopathic chemicals) from the leaves of *C. procera* using organic and aqueous solvents.
2. Germination percentage, seed vigour and mean seedling growth of the test plants crops, i.e. *Triticum aestivum* L., *Spinacia oleracea* L. and weeds, i.e. *Chenopodium album* L., *Cannabis sativa* L. with aqueous and organic solvents were assessed upon exposure of seeds of test plants.

9.2. METHODOLOGY

Fresh, healthy leaves of *C. procera* were plucked from plants growing in the University campus grouped into two halves. The first half was subjected to the recovery of aqueous leachates following the method given in Protocol I (Chapter 3, Materials and Methods). The leaves of the second half were shade dried and powdered. The powder was subjected to the organic extract fractionation employing various polar and non polar solvent systems following the method given in Protocol-II (Chapter 3, Materials and Methods).

The mature and viable seeds of *T. aestivum, S. oleracea, C. album and C. sativa* were used for the study. Aqueous leachates (AL) (g fresh wt/ml pure water 0.1% w/v), petroleum ether fraction (PF) (0.1% w/v), methanolic fraction (MF) (0.1% w/v), chloroform fraction (CF) (0.1% w/v) and water fraction (WF) (0.33% w/v) derived from leaves of *C. procera* formed the treatment solutions (Sisodia and Singh, 2012). The comparable treatment with pure water served as control in any of the cases. The concentrations of various fractions under experimentation were decided based on a pilot experiment on the % of germination where a wide range was used (Sisodia and Singh, 2012). The one that showed 50±10 percent values served as LC 50 (Lethal concentration) killing 50% of the population (Sisodia and Singh, 2012). For each of these treatments, five replicates were maintained. The data represent mean S.D. (standard deviation) of five sets.

9.3. GERMINATION PARAMETERS

Healthy, viable and uniform seeds with sample size of 300 of all species were taken under test as per methodology adopted by Sisodia and Singh (2012). Seeds of

each sample were treated with respective concentrations of treatment solution for 20 hours and maintained at room temperature. Distilled water used as a treatment served as control. The seeds were placed in Petri dishes with a thin layer of absorbent cotton covered with Whatman no. 40 filter paper and moistened with respective concentration of organic and aqueous solvents along with its maintenance in a seed germinator. Following ISTA rules (1976), the test for germination percentage and seed vigour was done. The root length and shoot length were measured after 7 days of germination of the seeds.

9.4. STATISTICAL ANALYSIS

Statistical analysis employed analysis of variance (ANOVA) on the parameters under study to evaluate the significance of difference of treatment with that of control.

9.5. RESULTS

The treated seeds completely failed to germinated in organic solvents with (87.17%) showing germination in water served as control in case of *T. aestivum* (Table 6.1). Germination percentage, radicle and plumule length also behaved similarly upon exposure to organic solvents. In control, about (70.18%) seed vigour was noticed in *T. aestivum*, with complete reduction (0%) in the treated samples (Fig. 6.1).

On third day, (90.49%) seed of *S. oleracea* germinated with water as a control. While the germination was completely inhibited upon exposure to organic solvents (aqueous leachates, petroleum ether fraction, methanolic fraction and chloroform fraction). However, in water fraction, about (5.58%) germination was noticed (Fig. 6.1). A very small emergence determined germination as visualized by hand lens. *S. oleracea* pronounced the seed vigour of (75.32%) in control which was dropped to zero by the treatment solution (Table 6.1).

Seeds of *C. album* showed germination of (70.06%) using water as a control on the second day. However, the germination was completely inhibited upon exposure to aqueous leachates, petroleum ether fraction and chloroform fraction (Fig. 6.2). However, upon exposure to methanolic fraction and water fraction germination percentage observed were very less. Further, (79.73%) of seed vigour of *C. album* was noticed in control, which was completely inhibited upon exposure to organic and aqueous solvents (Table 6.2).

Serving water as control, (80.74%) seeds germinated in *C. sativa.* While the germination was completely inhibited upon treatment with allelochemicals extracted with aqueous leachates, petroleum ether fraction and methanolic fraction. Further, (3%) and (9%) germination was exhibited by chloroform fraction and water fraction treatment, respectively. Seed vigour of *C. sativa* noticed was (80.56±0.42) (Table 6.2). Results pertaining to parameters showed high significance statistically (Fig 6.2).

Table 6.1: Response of germination behavior of (a) *T. aestivum* and (b) *S. oleracea* seeds upon exposure to aqueous leachates and organic fractions extracted from leaves of *C. procera.*

Treatment	(a) *T. aestivum*				(b) *S. oleracea*			
	Germination percentage (%)	**Root length (mm)**	**Shoot length (mm)**	**Seed vigour %**	**Germination percentage (%)**	**Root Length (mm)**	**Shoot length (mm)**	**Seed vigour %**
Control	87.17±0.35[a]	8.2±0.48[a]	13.63±0.36[a]	70.18±0.30[a]	90.49±0.31[a]	6.66±0.33[a]	10.71±0.23[a]	75.32±0.17[a]
Aqueous leachates AL (g/ml fresh wt.)	0	0	0	0	0	0	0	0
Petroleum ether fraction PF (0.1% w/v)	0	0	0	0	0	0	0	0
Methanolic fraction MF (0.1% w/v)	0	0	0	0	0	0	0	0
Chloroform fraction CF (0.1% w/v)	0	0	0	0	0	0	0	0
Water fraction WF (0.33% w/v)	0	0	0	0	5.58±0.13[b]	3.57±0.41[b]	3.57±0.41[b]	0
LSD at 5%	**0.37**	**0.49**	**0.37**	**0.31**	**0.35**	**0.54**	**0.48**	**0.17**

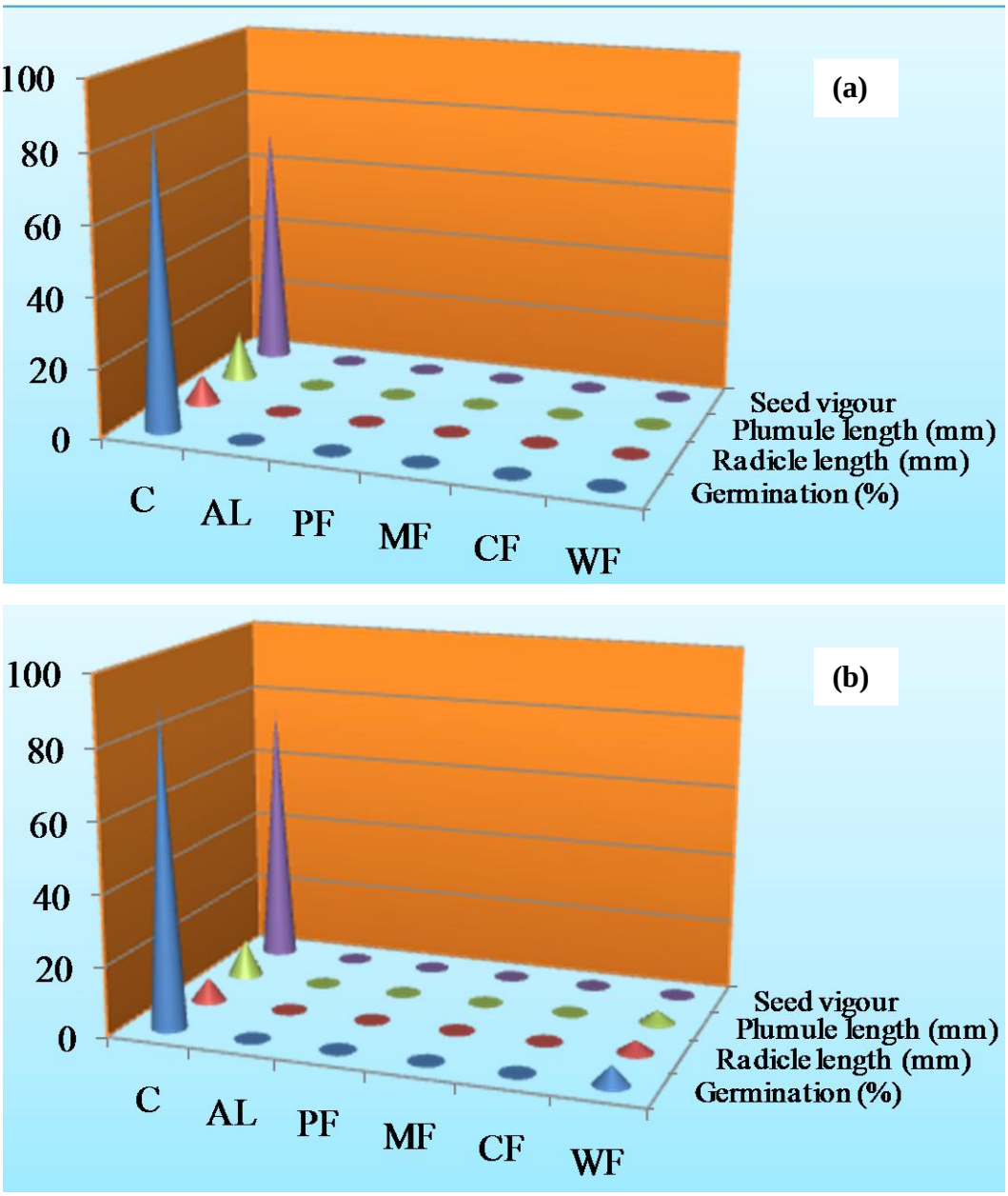
Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

Table 6.2: Response of germination behavior of (a) *C. album* and (b) *C. sativa* seeds upon exposure to aqueous leachates and organic fractions extracted from leaves of *C. procera.*

Treatment	(a) *C. album*				(b) *C. sativa*			
	Germination percentage (%)	**Root length (mm)**	**Shoot length (mm)**	**Seed vigour %**	**Germination percentage (%)**	**Root length (mm)**	**shoot length (mm)**	**Seed vigour %**
Control	70.06±20.42[a]	4.20±0.48[a]	9.58±0.34[a]	79.73 ±0.81[a]	80.74±0.52[a]	5.69±0.34[a]	11.48±0.48[a]	80.56±0.42[a]
Aqueous leachates AL (g/ml fresh wt.)	0	0	0	0	0	0	0	0
Petroleum ether fraction PF (0.1% w/v)	0	0	0	0	0	0	0	0
Methanolic fraction MF (0.1% w/v)	2.44±0.44[c]	1.10±0.10[c]	2.56±0.41[c]	0	0	0	0	0
Chloroform fraction CF (0.1% w/v)	0	0	0	0	3.27±0.35[c]	1.81±0.22[c]	4.98±0.12[c]	0
Water fraction WF (0.33% w/v)	7.5±0.38[b]	2.12±0.23[b]	7.80±0.18[b]	0	9.81±0.22[b]	3.5±0.19[b]	6.51±0.28[b]	0
LSD at 5%	**0.64**	**0.56**	**0.58**	**0.84**	**0.69**	**0.46**	**0.59**	**0.43**

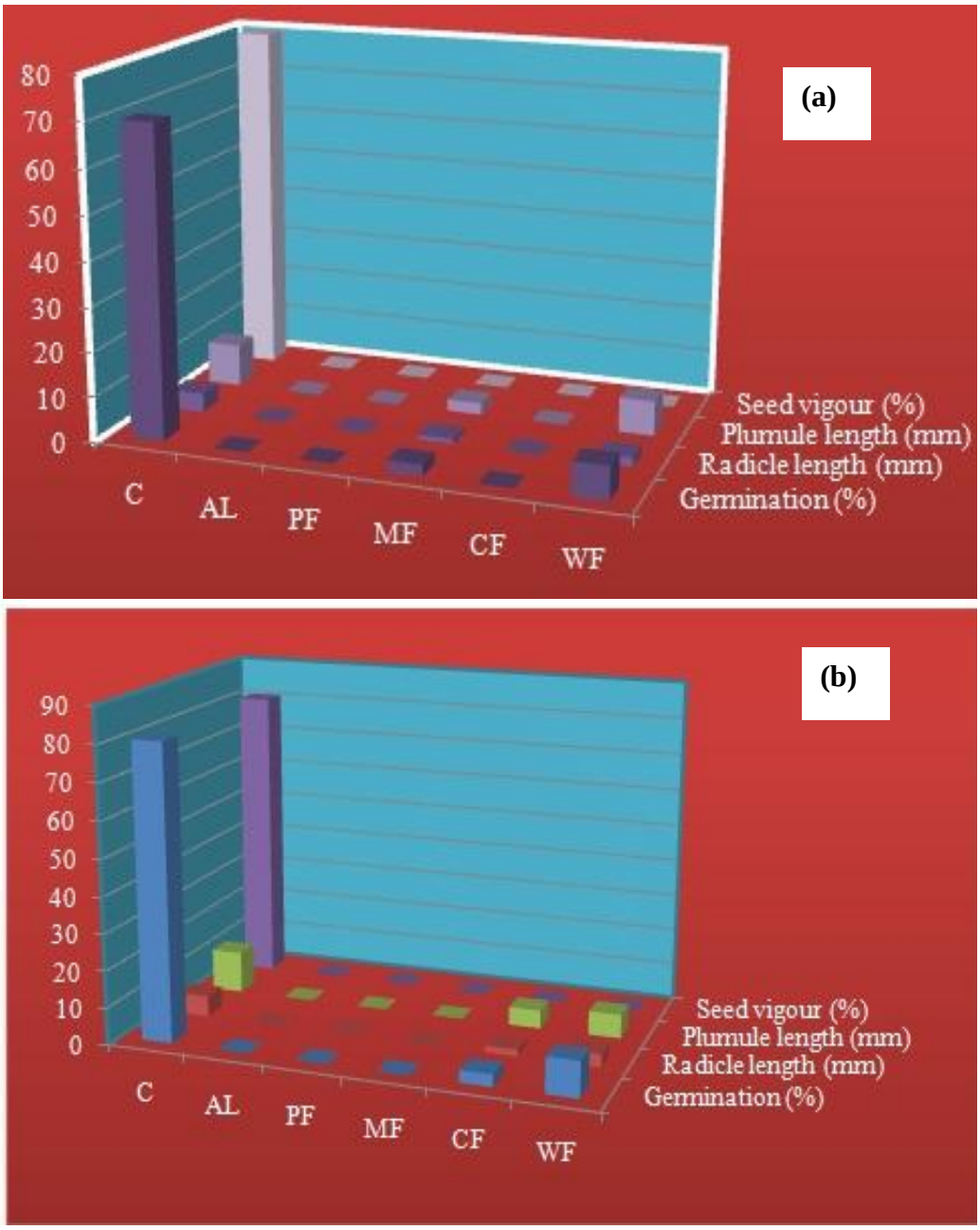
Different superscript symbols represent significant difference among themselves at P< 0.05 applying DMRT.
± represents standard deviation.

Fig. 6.1: Response of germination behavior of (a) *T. aestivum* and (b) *S. oleracea* seeds upon exposure to aqueous leachates and organic extract fractions from leaves of *C. procera.*



C = Control
AL = Aqueous leachates (g/ml fresh wt.)
PF = Petroleum fraction (0.1% w/v)
MF = Methanolic fraction (0.1% w/v)
CF = Chloroform fraction (0.1% w/v)
WF = Water fraction (0.33% w/v)

Fig. 6.2: Response of germination behavior of (a) *C. album* and (b *C. sativa* seeds upon exposure to aqueous leachates and organic extract fractions from leaves of *C. procera.*



C = Control
AL = Aqueous leachates (g/ml fresh wt.)
PF = Petroleum fraction (0.1% w/v)
MF = Methanolic fraction (0.1% w/v)
CF = Chloroform fraction (0.1% w/v)
WF = Water fraction (0.33% w/v)

9.6. DISCUSSION

Significant inhibition of germination dynamics and seedling growth of species tested upon analysis and interpretation of the results under the influence of the aqueous and organic fraction solvents as revealed by (Tanveer *et al.* 2012) supports our study. Differential level of phytotoxicity in response to aqueous extracts and organic fractions was exhibited against test species that might arise due to the variable chemical nature of the compounds used for extraction (Tanveer *et al.*, 2012). As stated by (Blair *et al*., 2009; Borghetti *et al*., 2013), the true allelopathy study intends to the purification and identification of the active substances involved in the observed interaction has been considered an essential step. Germination and growth of weeds and crops inhibited by leachates from leaf, shoot and flower of *C. procera* (Al-Zahrani and Al-Robai, 2007; Knox *et al*., 2010; Yasin *et al*., 2012; Abdel-Farid *et al*., 2013; Naz and Bano, 2013; Ayeni and Akinyede, 2014; Gomaa *et al*., 2014; Gulzar *et al*., 2014a,b; Gulzar *et al*., 2015a) also supports our finding. The allelopathic potential of *C. procera* also becomes evident from the results of the previous experiments.

The presence of water soluble inhibitors in *C. procera* extract may reflect the arrest of seed germination and germination percentage reduction (Yasin *et al*., 2012). The present work is in line with the study reported by (Tefera, 2002; Stavrianakou *et al.*, 2004, Dongre and Yadav, 2005; Kadioglue *et al*., 2005; Oyun, 2006; Siddiqui *et al*., 2009; Tanveer *et al*., 2010; Hussain *et al*., 2011; Ahmad, 2012; Novoa *et al*., 2012; Oluwole *et al*., 2013) who revealed suppression in the germination rate and final germination of the target species by allelopathic plants. Harmones especially gibberllins gets inhibited by allelochemical compounds lead to decrease of germination. Change in enzyme activities may also be responsible for the decrease of germination during germination period, which restricted the conversion of nutritive compounds (El-Khatib *et al*., 2004). In this study, reduced cell division and alteration in the ultrastructure of the cells induced by phenolic allelochemicals is correlated with a reduction in the seedling growth of the target species (Li *et al.*, 2010). As stated by (Rice, 1984, Kayode, 2006), exudation or volatilization or leaching are the various ways through which allelochemicals are leached into the environment. In glycosidic forms, allelochemicals or secondary metabolites are released into the environment (Sisodia and Singh, 2012). Glycosidic bonds, not only facilitate the movement of allelochemicals in and outside the plant, but also lessen their toxic nature towards the

donor plant itself (Goss, 1973; Sisodia and Singh, 2012). The presence of glycosides in the aqueous leachates of *C. procera* was confirmed by fehling solution test. The glycosides, however, could be separated from the organic part either by enzymatically action or by acid hydrolysis (Sisodia and Singh, 2012).

Water is a polar compound while chloroform, petroleum ether and methanol are non-polar in nature. Tanveer *et al*. (2012) stated that different extraction efficiencies of the solvents, lead to variable phytotoxicity of different aqueous and organic fractions accounting for qualitative and quantitative differences in extracted phytotoxins in different fractions. A similar case was also observed in our results. Reduction in germination and seedling growth exhibited by aqueous extract of *C. procera* reveals water as the best solvent to be used for extraction of phytotoxic compounds in our results. Whitehead *et al*. (1981); Li *et al*. (2010); Tanveer *et al*. (2012) concluded that phenolics, a major category of compounds responsible for allelopathic activity can best be extracted in water. The highest allelopathic potential was revealed by Javaid *et al*. (2010, 2011) and Tanveer *et al*. (2012)) while screening organic and aqueous solvents of *Withania somnifera* (L.) Dunal against *Parthenium hysterophorus* L., *Phalaris minor* Retz and that of *Euphorbia dracunculoides* Lam. against chickpea and wheat. This is of great ecological significance in allelopathic interference on behalf of water soluble compounds, particularly in cultivated fields that are infested with this weed and are frequently irrigated or receive rainwater (Tanveer *et al*., 2012). Khaliq *et al.* (2011) and Tanveer *et al*. (2012) confirmed that the differential inhibition by allelopathic products is in agreement with our results. Moreover, sample preparation and extraction techniques are believed to lead variation in magnitude of allelopathic suppression (Zielinski and Kozlowski, 2000; Javaid *et al*., 2011).

In fact, the inhibitory effect impacted by aqueous extracts on seedling growth was found to be much greater than was that achieved with the organic fractions as also stated by Borghetti *et al*. (2013). The use of organic solvents in the preparation of extracts should be avoided when the proposal of a study is to reveal some kind of allelopathic interaction taking place in the field (Blum, 2011; Borghetti *et al*., 2013). Further, in allelopathic interference the use of water has been recommended because it reflects more closely what would happen under natural conditions (Ferreira, 2004; Borghetti *et al*., 2013). However, once the allelopathic activity of a species has been

demonstrated, the solubilization of the active fractions in organic solvents may be desirable because it facilitates the subsequent steps of separation and identification of the active molecules (Macias *et al*., 2004).

Section VII

Allelochemical Identification

10. OBJECTIVE

To investigate profile of phenolics and isolate the different allelochemical compounds from different parts of *Calotropis procera* (Ait.) R. Br.

10.1. HYPOTHESIS TO BE TESTED

In the previous studies, it was observed *C. procera* exert a negative effect on crop and weed plants. This was attributed to the water-soluble phenolics released from different parts of *C. procera*. Since phenolics present a heterogeneous group of compounds of different chemical nature viz. coumarins, alkaloids, flavonoids and most common phenolic acids, an attempt was made to identify them. Based on literature survey, the presence of different phenolic acids that play an important role in allelopathy was checked. For this study, above ground parts (leaves and stem) and below ground part (roots) of *C. procera* were used.

10.2. PARAMETERS STUDIED

Phenolic acids were identified through High Performance Liquid Chromatography (HPLC) at Indian Institute of Integrative Medicine (IIIM), Srinagar. The retention time (RT) of different peaks from chromatograms obtained from extracts and authentic samples were recorded.

10.3. METHODOLOGY

10.3.1. Preparation of extracts for analysis through HPLC (Gulzar *et al*., 2015a)

A methanolic extract of the powdered leaf, stem and root was chemically analyzed by a HPLC System (LC-10A, SCL-10A, Shimadzu HPLC, Tokyo, Japan) for identification and quantification of suspected phytotoxins. Because of the high efficiency of pure methanol for hydrophilic compounds, it was used to extract the free phenolic acids from the soil (Kong *et al.*, 2006). Furthermore, the methanol has a protective role, because it can prevent phenolic compounds from being oxidized by enzymes, such as phenoloxidases (Proestos *et al.*, 2006). The separation conditions are listed in Table 7.1. However, once the allelopathic activity of a species has been demonstrated, the solubilization of the active fractions in organic solvents may be desirable because it facilitates the subsequent steps of separation and identification of the active molecules (Macias *et al.*, 2004). As reported by Borghetti *et al*. (2013), the procedure described here allows the purification of allelochemicals that are more polar, because it helps to remove from the aqueous solutions insoluble and undesirable substances that could hamper further chromatographic analysis, a useful

procedure if one intends to delve further into the study of allelopathy. Ten-gram powder of leaf, stem amd root was extracted with 80% methanol and shaken on a rotary shaker for 24 hours at room temperature. The supernatants were separated and their pH fixed to 2.0 with the help of 2M HCl. The solutions were extracted three times with 50 ml of ethylacetate. The resultant solutions were dried and evaporated to dryness on a rotary evaporator at 40°C. The phenolic acids were extracted by adding methanol to these condensed residues (obtained after evaporation) in such a way to contain the concentration of 1 mg/ml. These methanol extracts of different parts were subjected separately to HPLC for identification. Besides, doses of authentic samples of different phenolic acids (Sigma/Aldrich/Fluka made) were dissolved in methanol at a concentration of 1 mg/ml and run parallel for identification purpose. The detection of peaks was done by UV detector. The concentration of each isolated compound was determined by the following equation (Alsaadawi *et al*., 2011).

$$\textbf{Concentration} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard} \times \text{Dilution factor}$$

Table 7.1: HPLC conditions to determine allelochemicals in methanol extract of different parts of *C. procera.*

Parameter	Characteristic
Dimensions of column	25 cm length×4.6 mm diameter; particle size 5µm, Agilent, Zorbax Eclipse plus
Detector	SPD-M10A *VP* tunable UV detector
Detection	UV, 270nm
Rate of flow	0.5ml/min
Injection volume	2µL
Column type	RP- C-18
Mobile phase	2% acetonitrile; 80% methanol
Temperature	70^0C
Column software	EZ chrome software

10.4. STATISTICAL ANALYSIS

The whole experiment was repeated and the mean values of RT with standard deviations presented.

10.5. RESULTS

A number of phenolic compounds responsible for the inhibitory allelopathic activity of *C. procera* were identified in the present study (Fig. 7.1,7.2,7.3). Chemical analysis revealed the presence of phenolic acids in the methanolic extract of leaf, stem and root of *C. procera*.

The average values of the RT of samples extracted from different parts of *C. procera* were calculated and compared with those of authentic samples of phenolic acids. In all, phenolic acids, namely caffeic acid, gentistic acid, catechol, gallic acid, syringic acid, ellagic acid, resorcinol, *p*-coumaric acid, *p*-hydroxy benzoic acid, vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, pyrogallic acid, furoic acid and ferulic acid were identified from leaf, stem and root of *C. procera* (Table 7.1,7.2,7.3).

In the green leaves, nine phenolic acids were detected. These were caffeic acid (RT=2.17), gentistic acid (RT=2.35), catechol (RT=2.62), gallic acid (RT=4.26), syringic cid (RT=5.72), ellagic acid (RT=6.94), resorcinol (RT=11.01), *p*-coumaric cid (RT=12.57) and *p*-hydroxy benzoic acid (RT=16.29) with different retention time and quantities (Fig. 7.1, Table 7.1).

Although eight phenolic acids [vanillic acid (RT=3.30, 3.27), chlorogenic acid (RT=4.52, 4.52), protocatecheic acid (RT=4.74, 4.65), quercetin (RT=4.93, 4.93), syringic acid (RT=5.38, 5.38), gallic acid (RT=6.72, 6.82), pyrogallic acid (RT=9.37, 9.04) and *p*-coumaric acid (RT=12.58, 12.4)] detected in stem and root extract were similar, however they show difference in terms of retention time and quantities that reflect their differential pytotoxicity. However, the furoic acid (RT=10.70) in stem extract and ferulic acid (RT=7.11) in root extract were identified different ones with their respective retention time and quantities in addition to these eight phenolic acids (Table 7.1,7.2,7.3).

Fig. 7.1: HPLC chromatogram of methanolic leaf extract of *C. procera.*

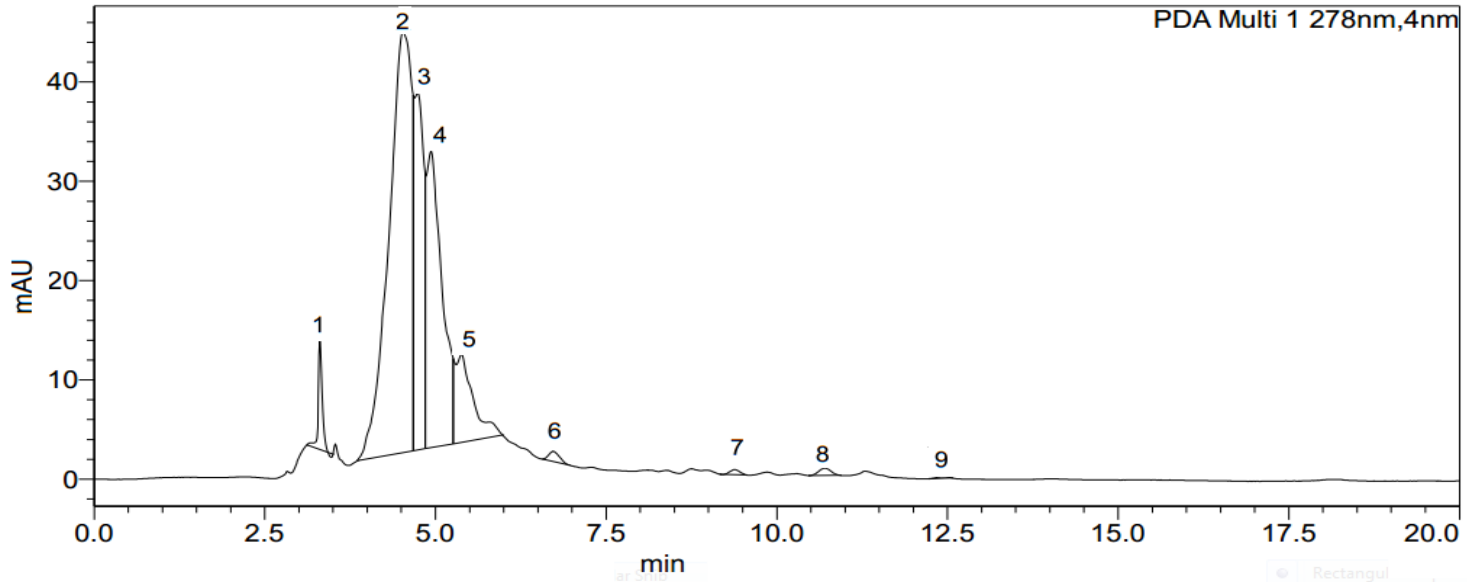


Table 7.1: Allelochemicals identified from methanolic extract of *C. procera* leaf (Gulzar *et al*., 2015a)

No.	Compound	Retention time	Area	Height	Area%	Lamda max
1	Caffeic acid	2.17	716125	89128	16.62	202/279/656/485/581
2	Gentistic acid	2.35	492349	73352	11.43	205/279/426/656/581
3	Catechol	2.62	1765904	168424	40.98	206/279/656/422/486
4	Gallic acid	4.26	759752	24557	17.63	199/254/374/484/685
5	Syringic acid	5.72	41800	4115	0.97	199/257/426/581/672
6	Ellagic acid	6.94	216088	14830	5.01	199/253/484/581/685
7	Resorcinol	11.01	41684	1484	0.96	199/273/255/484/306
8	*p*-coumaric acid	12.57	-135	4	-0.003	199/272/484/581/639
9	*p*-hydroxy benzoic acid	16.29	273719	960	6.35	199/277/484/309/581

Fig.7 2: HPLC chromatogram of methanolic stem extract of *C. procera.*

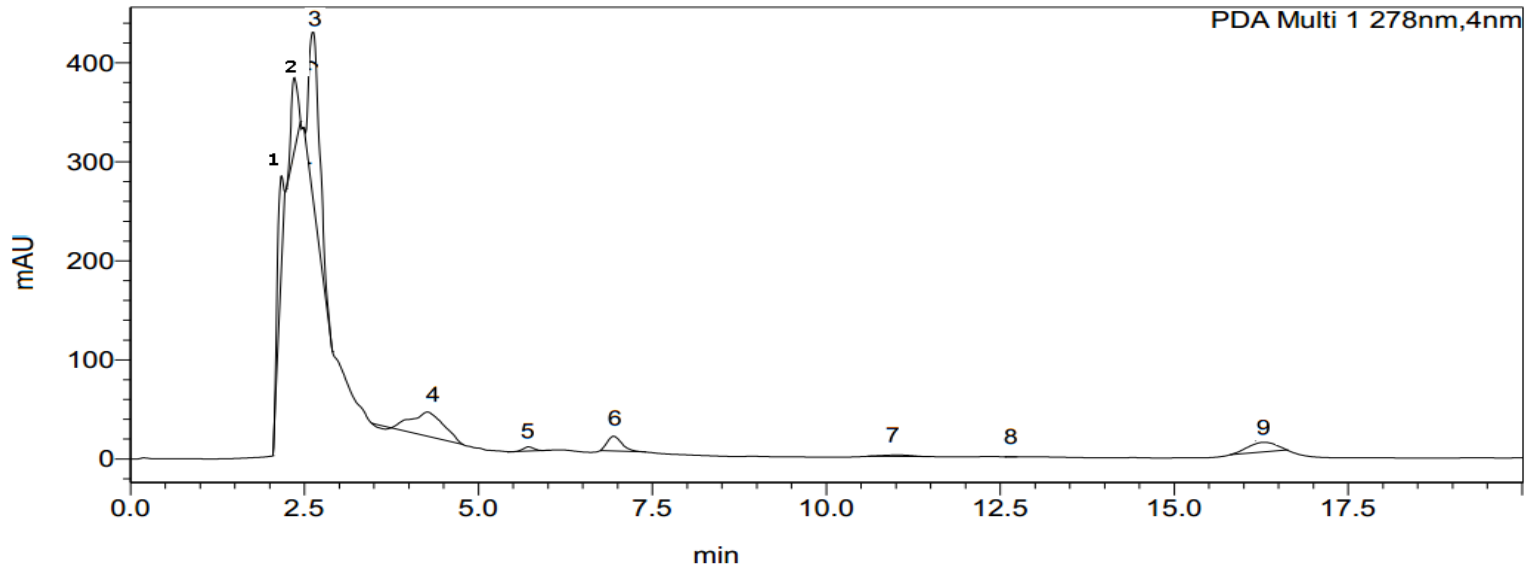


Table 7.2: Allelochemicals identified from methanolic extract of *C. procera* stem.

No.	Compound	Retention time	Area	Height	Area%	Lamda max
1	Vanillic acid	3.30	43534	11136	2.118	204/279/487/640/664
2	Chlorogenic acid	4.52	981449	42448	47.760	204/276/657/470/640
3	Protocatecheic acid	4.74	362066	35948	17.619	204/276/657/582/470
4	Quercetin	4.93	473930	29834	23.063	204/278/656/470/640
5	Syringic acid	5.38	167175	8845	8.135	204/279/656/429/470
6	Gallic acid	6.72	11209	1033	0.545	210/195/312/656/470
7	Pyrogallic acid	9.37	4881	504	0.238	236/656/287/444/582
8	Furoic acid	10.70	9005	726	0.438	656/279/245/487/381
9	*p*-coumaric acid	12.41	1723	157	0.084	205/656/279/381/582

Fig.7. 3: HPLC chromatogram of methanolic root extract of *C. procera.*

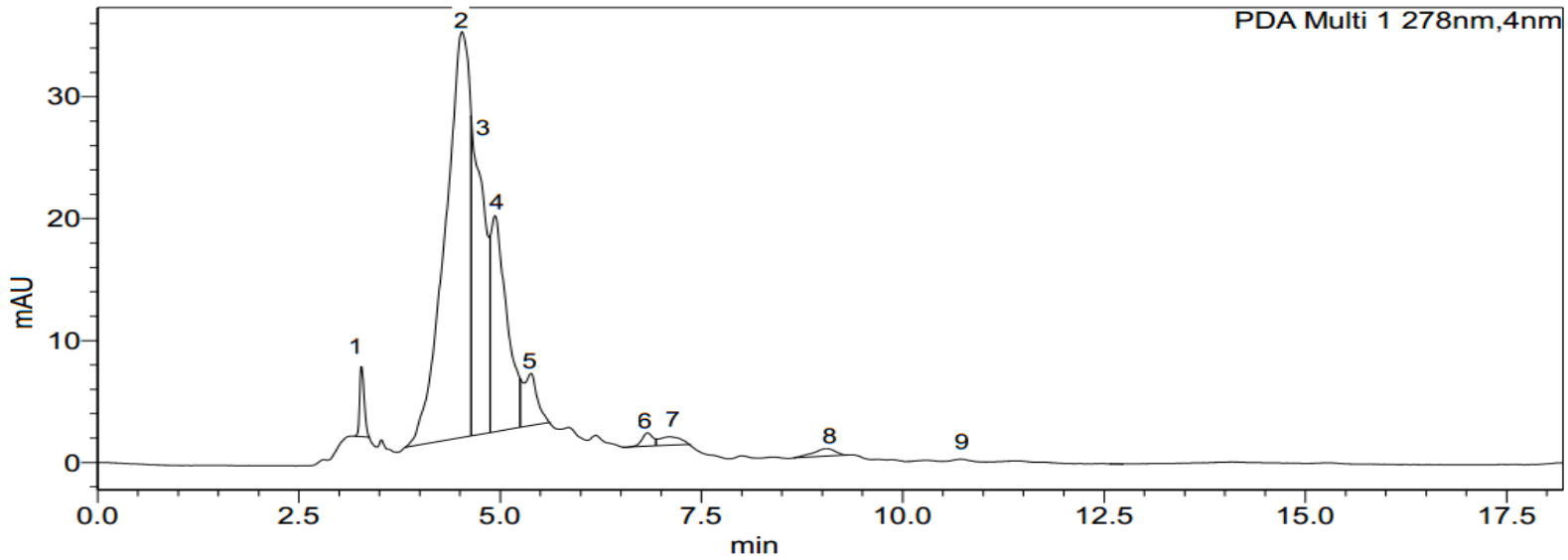


Table 7.3: Allelochemicals identified from methanolic extract of *C. procera* root

No.	Compound	Retention time	Area	Height	Area%	Lamda max
1	Vanillic acid	3.27	22243	5930	1.660	204/280/479/582
2	Chlorogenic acid	4.52	704644	33271	52.574	204/276/656/467/582
3	Protocatecheic acid	4.65	283641	25116	21.163	204/275/487/675/636
4	Quercetin	4.93	241236	17727	17.999	203/278/656/467
5	Syringic acid	5.38	53678	4281	4.005	204/278/656/487
6	Gallic acid	6.82	10249	1088	0.765	203/283/656/400/487
7	Ferulic acid	7.11	12969	695	0.968	204/283/656/400
8	Pyrogallic acid	9.04	11613	616	0.866	283/656/203/467
9	*p*-coumaric acid	12.58	17	7	0.001	204/656/582/384/467

10.6. DISCUSSION

The allelochemicals of *C. procera* were identified to be phenolic acids. The presence of phenolic acids in the different parts of *C. procera* indicates that these play an important role in imparting phytotoxic/allelopathic property to this weed. All the identified phenolic acids are known allelochemicals (Rice, 1984). Chromatographic analysis provided additional insights into phytotoxin-mediated suppression of the tested species tested by determining the qualitative and the quantitative presence of different compounds (Tanveer *et al*., 2012). Extensive research needs to be conducted on phenolic compounds regarding their mode of action as plant growth inhibitors. Most of these compounds are water-soluble and when present in sufficient concentration can cause allelopathic activity in their immediate vicinity (Tanveer *et al*., 2012). These compounds are known to interfere with basic structures and functions in the tested species, causing various forms of stress resulting in impaired germination and diminished growth (Duke and Dayan, 2006). They influence nutrient uptake, membrane permeability, protein synthesis, photosynthesis, respiration, enzyme activity, hormone balance and water potential (Li *et al*., 2010).

Phenolic acids are predominantly found in allelopathic plants and are synthesized within the plants as secondary metabolites (Gulzar *et al.,* 2015a). Within plant, remain in the glycosidic form to avoid intra-plant toxicity and/or facilitate movement within and outside the plant. In fact, numerous secondary metabolites are produced by plants, some of them show growth inhibitory effects on other plants such as allelopathic activity (Duke *et al*., 2000b). Some plant species provide excellent weed control in intercropping or as soil additives (Caamal-Maldonado *et al*., 2001; Kato-Noguch *et al*., 2014). Allelopathic substances have the potential either as herbicides or as templates for new synthetic herbicide classes (Duke *et al*., 2000b; Omezzine *et al*., 2014b). In the last three decades, the potential of phenolic acids as allelochemicals has been widely described in the literature, not only in laboratory bioassays, but also in field studies (Chung *et al*., 2002; Inderjit *et al*., 2002; Iqbal *et al*., 2003; Beninger *et al*., 2004; Djurdjevic *et al*., 2004; Sanchez-Moreiras *et al*., 2004; Kim *et al*., 2005; Blum and Gerig, 2005; Baratelli *et al*., 2012; Gulzar *et al*., 2015a; Ren *et al.,* 2015; Liu *et al*., 2016). These allelochemicals are, particularly, prone to qualitative and quantitative variations, depending on genetic drift and ploidy level (Te Beest *et al*., 2011), physiological conditions, season, harvesting time and

analytical method sample preparation (Cirak *et al.,* 2008). The phenological stage is another source of variability that considerably influences the secondary metabolite concentrations (Cirak *et al*., 2008; Omezzine *et al*., 2014b). Because of their different polarities, the amounts of compounds in the extracts will differ and these differences could explain their toxicity (Omezzine *et al*., 2014b). The qualitative differences in the composition of phenolic compounds in the methanolic extracts in different parts of plant could contribute to the differences of phytotoxicity effect (Omezzine *et al*., 2014b). The presence of the phenolic acids in leaf, stem and root extract, suggests allelopathic interference; however, leaf extract noticed in various experiments exhibited greater biological activities. This is due to difference in quantities of allelochemicals extracted from different parts which reflect their differential behavior. It is possible that the different extraction procedure caused an extraction of the same main substances, but in different concentrations and subsequent different levels of biological activity (Macias *et al*., 1997; Chon *et al.*, 2005; Almeida and Delachiave, 2008). This is confirmed in our experiments, where the nine compounds were present in leaf, stem and root methanolic extract in quantities that differ subsequently.

Section VIII
Foliar Micromorphology

11. OBJECTIVE

To study the link between allelopathic potential of water soluble allelochemicals and trichomes on leaf surfaces of *Calotropis procera* (Ait.) R. Br. and their contribution to phytotoxicity.

11.1. OBSERVATION PARAMETERS

The following observations were made:

1. Scanning electron microscopy of leaf surfaces of *C. procera.*
2. The contribution of phenolic allelochemicals located on the leaf surfaces of *C. procera* to the suppressed and development of seedlings of *Triticum aestivum* L. was examined.
3. Scanning electron microscopy (SEM) of sections of dipped leaves prepared to investigate the leaf surface before and after dipping in an organic solvent in order to detect the possible changes to the trichomes.

11.2. MATERIALS AND METHODS

11.2.1. Collection of the material for scanning electron microscopy

The varying magnifications of the adaxial and abaxial surfaces of *C. procera* were performed by using the JEOL (JSM-6510LV) SEM (Plate 5A) operated at 10-15 keV acceleration voltage. The *C. procera* was harvested during its vegetative stage from a natural population around the campus of Aligarh Muslim University, Aligarh. Plant identification was done by an expert (Plant taxonomist) and a voucher no. 541 was deposited in the herbarium of Department of Botany, A.M.U., Aligarh. The methodology adopted by (Vaishali *et al*., 2008; Badmus, 2012; Wintola and Afolayan, 2014; Gulzar *et al.*, 2015a) was used to examine the foliar ultramorphology following the general procedures. Freshly cut leaf samples were rinsed in distilled water and sectioned into about 4-6 mm segments before fixing in 0.05M sodium cacodylate and rinsed again in 0.05M cacodylate buffer (pH 7.5). Dehydration of the samples was performed by passing through a graded series of ethanol (20%-100%) three times at 20 min. per rinse. This was followed by critical point drying with liquid carbon-dioxide in Hitachi HCP-2 Critical Point Dryer (Plate 5B). For mounting of each dried sample, aluminum specimen stubs with double sided carbon coated adhesive discs and sputter coated with gold palladium (Eiko IB-3 Ion Coater) was preferred. The JEOL (JSM-6510LV) SEM were operated at 10-15keV for examining adaxial and abaxial surfaces of the leaf specimen at varying magnifications. All the

representative features examined were captured digitally using Microsoft Image Software for windows (Wintola and Afolayan, 2014)

11.2.2. Leaf-dipping experiments

Bioassays for determining the effect of the metabolites exclusively located on the leaf surface of *C. procera* on germination and seedling development of *T. aestivum* were done by using dichloromethane (organic solvent) with different concentrations.

11.2.2.1. Bioassay 1: Leaf dipping for five seconds

A collection of four fresh leaves, weighing 4 g were done from *C. procera* in the mature stage. Material from the leaf surface and the trichomes were collected by rinsing with dichloromethane (DCM). This type of extraction has been previously found to almost completely extract the compounds (Duke *et al*., 1994, 1999; Tellez *et al*., 1999; Morimoto *et al.,* 2009). These leaves were subsequently dipped in 100 ml dichloromethane [This relates to preparation of aqueous extract from previous (Chapter-4, Section II) 4 g leaf powder in 100 ml of distilled water) for five seconds to obtain a full strength (100%) solution. Care was taken while dipping each leaf that leaf petiole not to be immersed in the organic solvent. The dilution of full strength (100%) solution was done to make up concentrations of 25%,50% and 75%. For control treatment, pure dichloromethane was used. The bioassay was conducted in Petri dishes (15 cm diameter) lined with one layer of Whatman No. 1 filter paper and wetted with 5 ml of the respective test solutions (Cusati *et al*., 2015). After evaporation of dichloromethane in a laminar flow cabinet, the Petri dishes were supplied with ten ml of distilled water and ten seeds of *T. aestivum.* After sealing the Petri dishes with parafilm, the setup was placed in the growth chamber at a temperature ±26^{o}C for 8 days. The root length and shoot length of seedlings were determined after eight days. Five replications were used for each extract concentration.

11.2.2.2. Bioassay 2: Leaf dipping for ten seconds

Ten intact C. *procer*a leaves weighing (4 g) were collected followed by separately dipping each leaf in 100 ml dichloromethane for 10 seconds to prepare a full strength extract (4 g/100ml, i.e. 100%). Care was taken not to include the petiole of the leaves while dipping in the solvent. The other dilutions (25%,50% and 75%) were then prepared by using dichloromethane. For the control treatment,

dichloromethane only was used. Bioassays were conducted in glass Petri dishes (15 cm diameter) lined with one layer of Whatman No. 1 filter paper and wetted with 5 ml of the respective test solution (Cusati *et al*., 2015). After evaporation of dichloromethane in a laminar flow cabinet, the Petri dishes were supplied with ten ml of distilled water and ten seeds of *T. aestivum.* After sealing the Petri dishes with parafilm, the setup was placed in the growth chamber at ±26^{o}C for 8 days. The root length and shoot length of seedlings were determined after eight days. Five replications were used for each extract concentration.

11.2.2.3. Bioassay 3: Ten second dipping in dichloromethane, followed by 24 hours in distilled water

The dipping procedure as described above was followed with 10 leaves (4 g) in dichloromethane for ten seconds and soaking in distilled water for 24 hours.

11.2.2.4 Bioassay 4: Leaf soaking in distilled water for 24 hours

This is the control or standard bioassay for evaluating the allelopathic potential of *C. procera.* Ten intact *C. procera* leaves (4 g) were soaked in a beaker filled with distilled water (100 ml), stirred, covered and left undisturbed in a dark place for 24 hours. After 24 hours, remaining concentrations (25%,50%,75% and 100%) were prepared by dilution of infusion. Five ml of infusion and ten wheat seeds was added onto the filter paper. Five replications of each concentration were prepared. After sealing the Petri dishes with parafilm, they were placed in a dark growth chamber at ±26^{o}C for eight days. The root and shoot length of each plant were measured after eight days.

11.3. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed in conjunction with each bioassay. Thus SEM was done on leaf sections of leaves that were:

1. Dipped in dichloromethane for five seconds.
2. Dipped in dichloromethane for ten seconds.
3. Dipped in dichloromethane for ten seconds, then soaked in distilled water for 24 hours.
4. Soaked in distilled water for 24 hours.
5. Fresh, undipped leaves were used as a SEM control comparison.

The intact leaves from each bioassay were frozen before performing their scanning electron microscopy. They were taken to the SEM section of USIF, A.M.U., Aligarh

for work to begin. The selected leaf portions of approximately 3×5 mm were exercised from the middle of the laminas between the midrib and leaf margin for scanning electron microscopy (SEM). SEM Autoclaving unit E5200 were used for coating the stubbed exposed leaf surfaces with gold after mounting. Collodal carbon was placed on the edges of the leaves on top of the gold coating. For conductive purposes and as a glue, collodal carbon was used to conduct excess electrons away from the areas to be examined. Observations and photographs were made using a JEOL (JSM-6510LV) SEM.

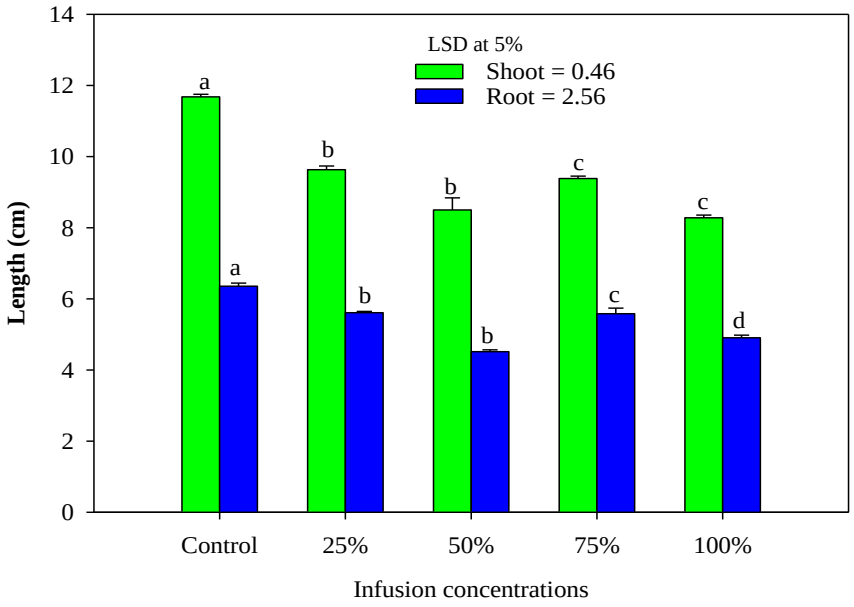
11.4. Results

On both the adaxial and abaxial leaf surfaces (Gairola *et al*., 2009), sessile glandular and non glandular trichome and stomata were found (Plate 8.1). These trichomes may or may not be the site for storage of allelopathic compounds or secondary metabolites produced by the plant.

11.4.1. Bioassay 1

Exposure of the leaf to dichloromethane for five seconds to show differences in structure and morphology of trichome, leaf epidermal cells and cuticle on upper and lower surface as revealed by SEM (Plate 8.2). The trichomes on upper surface seems more deflated with no change on epidermal structure. While on lower surface, the trichome seems no longer affected. The epidermal cells of lower surface undergo deformation resulting in rough surface. The results of this bioassay did not show any specific growth trend (hormesis). The root length shows more sensitivity than shoot length (Fig. 8.1).

Fig. 8.1: **Root length and shoot length of wheat seedlings exposed to different dichloromethane solutions prepared by soaking *C. procera* leaves for five seconds – Bioassay 1.**

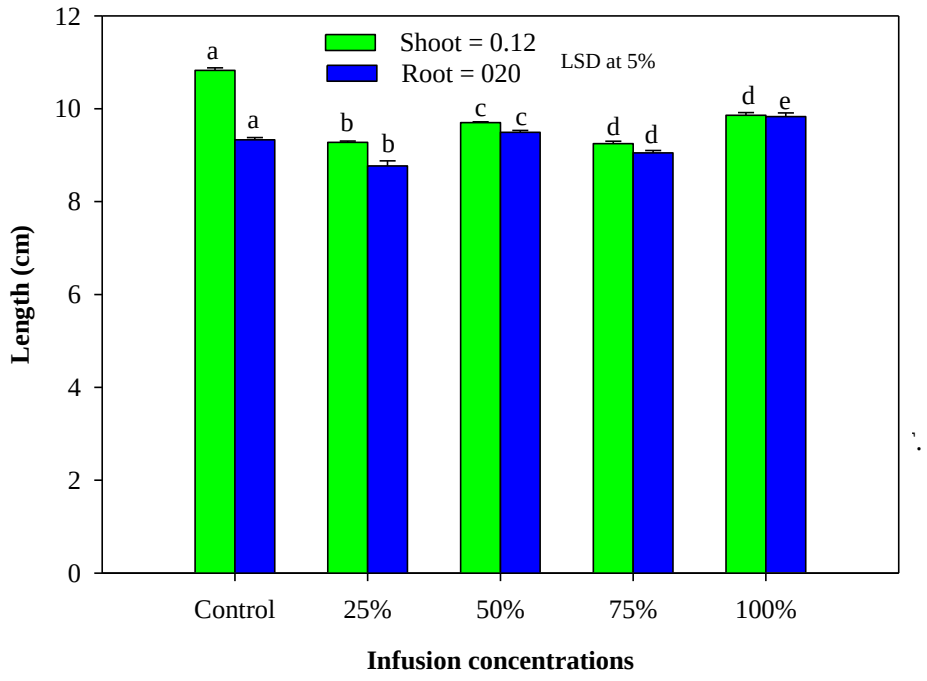


Means followed by same letters are not significantly different applying DMRT with bars representing standard deviation.

11.4.2. Bioassay 2

Although no clear tendency in terms of seedling growth inhibition or hormesis was detected (Fig. 8.2). No clear significant differences were found in bioassay 2. But SEM shows (Plate 8.3) that almost all of the trichomes on lower leaf surfaces exposed for ten seconds in dichloromethane get flaccid with only few in turgid state. The flaccid trichomes seems as a papery structure lying on the leaf surface. The shape of the stomata also distorted on lower surfaces with no pronounced change on leaf surface. On the upper leaf surface, the trichomes also seems flaccid. However, no such change was detected on the leaf surface and in stomata.

Fig 8.2: **Root length and shoot length of wheat seedlings exposed to different dichloromethane solutions prepared by soaking *C. procera* leaves for ten seconds – Bioassay 2.**

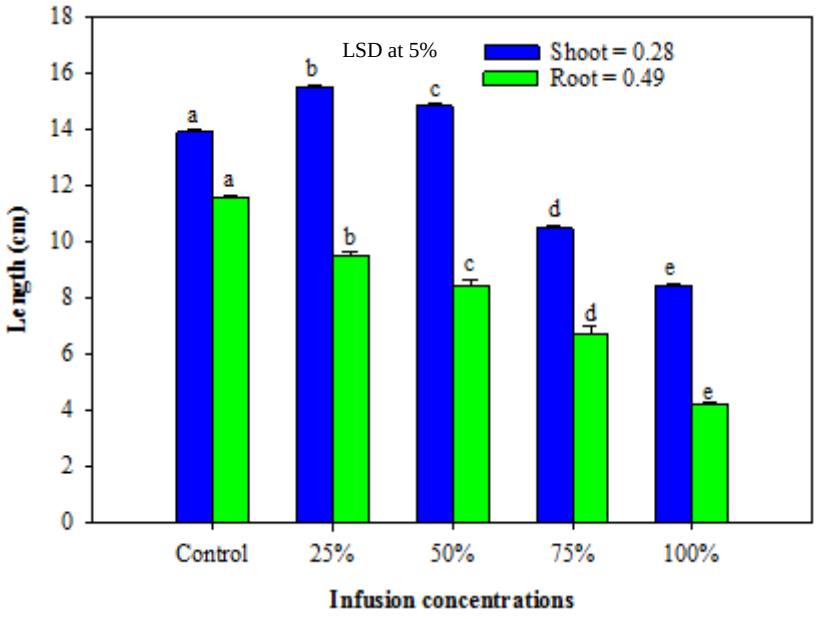


Means followed by same letters are not significantly different applying DMRT with bars representing standard deviation.

11.4.3. Bioassay 3

From the Fig. 8.3, it can be seen that *Triticum* shoots showed a typical hormesis response to the aqueous infusion prepared after leaves were dipped in dichloromethane. The roots, however showed a progressive decrease in growth with an increase in infusion concentration. This finding points to water-soluble allelochemicals being responsible for the allelopathic potential displayed by *C. procera*. The effect of dipping the *C. procera* leaf for ten seconds and then soaking it for 24 hours in distilled water can be seen in Plate 8.4. The trichomes seem flaccid releasing the contents along with the alteration in the epidermal cell walls and stomata shape. The cells of epidermis shrinkan. Besides the trichomes shows breakage as is shown by an arrow (Plate 8.4A), which might be the site for release of allelochemicals. All trichomes are shrinked along with the closure of stomata.

Fig. 8.3: Root length and shoot length of wheat seedlings exposed to different dichloromethane solutions prepared by dipping *C. procera* leaves for ten seconds and then soaking in distilled water for 24 hours– Bioassay 3.

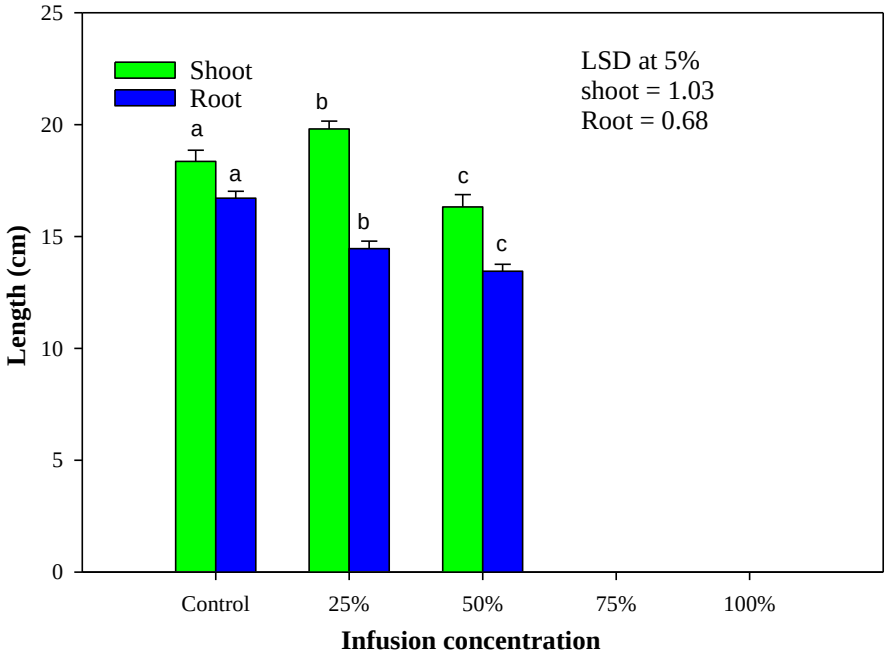


Means followed by same letters are not significantly different applying DMRT with bars representing standard deviation.

11.4.4. Bioassay 4

In this bioassay (Fig. 8.4), there was stimulation at lower concentrations and inhibition at higher concentration of seedling growth (hormesis). The effect on root length was greater than on shoot length, thus suggesting that roots were more sensitive to *C. procera* allelochemicals. Plate 8.5 shows greater changes in the structure of trichome by soaking the *Calotropis* leaf in distilled water. The trichome seems fully flaccid, lying attached to the epidermal surface. It seems all allelochemicals present in trichome are released into distilled water that bring about the inhibitory effect.

Fig. 8.4: Root length and shoot length of wheat seedlings exposed to different infusion concentrations of *C. procera* after soaking leaves in distilled water for 24 hours – Bioassay 4.



Means followed by same letters are not significantly different applying DMRT with bars representing standard deviation.

Plate 8.1 shows the SEM of leaf sections neither soaked in distilled water nor in dichloromethane. The sitting trichomes looks complete with absolutely no damage appearing on the leaf surface of the leaf, although the separation of upper part of trichome from the rest of trichome contributes the allelochemicals to seedling growth inhibition at the site of breakage. There was no change on the surface of the leaf on exposure to distilled water for 24 hours. However, platelet like structures occurs on the leaf surfaces, which needs their further confirmation through GC-MS chromatography.

11.5. Discussion

Once it was determined that leaves held the strongest allelopathic potential followed by root and stem in previous studies. Both sessile glandular and non-glandular trichomes were present in *C. procera* that is in line with the study of (Gabr *et al*., 2015). Glandular trichomes are bicellular with uniseriate stalk and unicellular head where as non glandular trichomes are with multicellular long broad and acute apical cell reported by Gabr *et al*. (2015). Electron microscopy was performed to determine whether allelopathic substances originate and/or stored on leaf surface. Some secondary compounds released from trichomes have also been implicated in allelopathy (Lovett, 1982), the stimulatory or inhibitory effects that chemicals produced by one plant may exert on another (Rice, 1984). For example, the leaves of *Heterotheca subaxillaris* (Lam.) Britt. & Rusby contain calamenene-type sesquiterpenes that inhibited the growth of *Agrostis stolonigfera*, *Lactuca sativa* and *Lemna pausicostata* (Morimoto *et al.,* 2009). Similarly, leaves of *Compuloclinium macrocephalum* (Less) D.C. bear glandular trichomes which excrete water soluble phenolic acids that reduced the growth of *Lactuca sativa* (Dixon, 2008). Positive results proved that trichomes are possible sources of allelochemicals on both adaxial and abaxial surfaces of young and mature leaves of *C. procera* that correlate with the recent study (Adedeji and Jewola, 2008; Dixon, 2008; Gairola *et al*., 2009; Badmus, 2012). A dipping experiment involving dichloromethane then followed to determine the solubility of the contents of trichomes found on the leaf surface**.**

Glandular trichomes secrete phytotoxins that exhibit the allelopathic interference. For example, allelochemical 1,8-cineole involved in plant-plant allelopathy (Muller and Muller, 1964) shows its presence almost entirely, if not entirely in the glandular trichomes of *Artemisia annua* (Tellez *et al*., 1999; Oliva and Duke, 2003). Similarly, other phytotoxic compounds such as terpenes are also localized in the glandular trichomes of *Salvia* species (Croteau and Johnson, 1984; Serrato-Valenti *et al*., 1997) that inhibit their recipient species with these and other terpenoid compounds (Muller and Muller, 1964; Muller *et al*., 1968; Duke *et al*., 1994). Such compounds reach to their competitors through volatilization from ground litter. Likewise, glandular trichomes of *Artemisia annua* contain the allelochemical artemisinin (Klayman, 1985) has been reported to be allelopathic (Duke *et al*., 1987, Chen and leather, 1990; Dayan *et al*., 1999) towards other plant species in the field,

even though mixing dried leaves in the soil suppresses growth of other plants in soil. Besides, all the plant parts including trichomes and pollen contain several secondary metabolites such as alkaloids, parthenin, kaempferol, *p*-coumaric acid and caffeic acid being high in leaves followed by the inflorescence, fruit, root and stem has been revealed by the chemical analysis of *Parthenium hysterophorus* (Kanchan, 1975; Patil and Hegde, 1988; Kapoor, 2012). The flavonoid allelochemicals present in high density in young leaves and/or near to the reproductive organs of *Paulownia tomentosa* (Thunb.) Steud was higher than in mature leaves (Kobayashi *et al*., 2008). The phytotoxins present in trichomes exhibit allelopathic interference on germination parameters and seedling growth has been reported by (Singh *et al*., 2006; Vrchotova *et al*., 2011; Lakmichi *et al*., 2010; Won *et al*., 2013) that correlates with our results in general.

The results of bioassay 1 did not show any specific growth tendency, i.e. hormesis or growth inhibition induced by allelochemicals released from trichomes are in line with the results of Belz and Hurle (2004); Dixon (2008). This does not correspond with findings by Kraus (2003) from similar experiments on *Parthenium hysterophorus*, where high biological activity was found in similar bioassays when leaves were dipped in the organic solvent, dichloromethane.

In a leaf washing (dipping) experiment on *P. hysterophorus* reported by Reinhardt *et al*. (2004) leaves dipped in TBME yielded up to 13.4 mg/g of the allelochemical perthenin where as the aqueous extract of leaves gave only 1.3 mg/g. This was not the case with *C. procera* as seen in Fig. 1 and Plate 2 where the dichloromethane had little effect on the leaf surfaces and trichomes, which perhaps explains why no allelochemicals were apparently released into the dichloromethane after five seconds.

Duke *et al.* (1994) found that dipping leaves of *Artemisia annua* into chloroform for a few seconds removed the contents of the peltate glands without causing any structural damage other than collapsing the cuticle covering these glands. Reinhardt *et al*. (2004) found similar effects (collapsed glands) in a study on *P. hysterophorus* when the contents of capitates sessile glands were removed using dichloromethane. SEM also showed a slit in the cell wall of capitates sessile glands on *P. hysterophorus* leaves with other trichomes appearing relatively unaffected. Kraus (2003) showed high biological activity towards test species in bioassays using these

extracts. This was not the case with *C. procera*. Figs. 8.1 and 8.2 show no specific growth tendencies, specifically hormesis-an important indicator of the allelopathic potential of a plant (Belz and Hurle, 2004). Thus, it is assumed that allelochemicals are either found on the leaf surfaces of *C. procera* or they must be insoluble in the organic solvent dichloromethane.

The appearance of breakage in one of the trichome might be the site of release of allelochemicals shown in Plate 8.4. However, further work is needed to confirm structure and function of trichomes. The effect where the organic solvent split the cuticle covering the capitates sessile trichome was also seen in experiments by Duke *et al*. (1994) on *A. annua* and by Reinhardt *et al.* (2004) on *P. hysterophorus.*

Plate 8.1 shows a leaf that was neither soaked in water nor dichloromethane. The trichomes looks complete with absolutely no damage appearing on the surface of the leaf. Results of experiments on *P. hysterophorus* (Reinhardt *et al*., 2004) showed that aqueous extracts of leaves yielded less parthenin (mg/g) than when dipped in the organic solvent TBME. Extraction for a 48-hour period only yielded slightly more parthenin compared to a 24 hour extraction period. In addition, the water solubility of parthenin was low. As can be seen from the results presented above, allelochemicals present on the leaf surfaces or in leaf tissue of *C. procera* have a higher water solubility than organic solvent solubility, making the findings of this experiment different to those of Reinhardt *et al*., (2004) and Duke *et al*., (1994). Picman and Picman (1984) suggest that in *P. hysterophorus*, the water soluble phenolics play important roles not only in allelopathy but also provides a defence mechanism against predators and disease and as autotoxins in regulation of population and the germination process timing. In nature, rain, fog, dew and mist can facilitate movement of water-soluble secondary metabolites from the plant to the immediate environment where they may persist to negatively affect the surrounding vegetation (Kohli *et al*., 1996).

Therefore, based on this observation following conclusion can be made:

- Structures found on the leaves of the plant could possibly contain the allelochemicals used by the plant to ensure its successful invasion growth.
- Results concurred that that the allelochemicals causing allelopathic potential of *C. procera* are probably water soluble (polar compounds).

- Allelopathic repercussions could happen once the plant has died and decaying matter in the grass field becomes wet in rainy seasons.

Section IX
Cytomorphology

Chapter 5
Discussion

DISCUSSION

Weeds constantly compete with crop plants to cause a considerable loss in their productivity (Jabran *et al*., 2015). Crop productivity can be lowered by weeds on an average of 34% (Oerke, 2006). The potential yield reductions from weeds in some important crops are: wheat 23%, soybeans 37%, rice 37%, maize 40%, cotton 36%, and potatoes 30% (Oerke, 2006). They act as a shield for the crop plants for available nutrients, space, light and moisture. Hence, in the presence of weeds physiological activities and growth of crops are negatively affected (Rajcan and Swanton, 2001). In addition, they deteriorate crop quality, clog waterways, cause health problems in humans and look unsightly in amenity areas such as garden, parks, pathways and pavements, etc. (Singh *et al*., 2003c). Weeds also cause fire hazards, besides being determinable to crop yields and unappealing (Zimdahl, 1999; Singh *et al*., 2003c). Weeds also are the permanent hosts of insects and pathogens, adding more complications to their control (Singh *et al*., 2003c). Hence, since the ancient times, weeds have been documented as serious plant pests (Zimdahl, 2013). Weeds have always played a role throughout the domestication of crop plants, which necessitated practicing weed control measures (Oerke *et al*., 1999; Zimdahl, 2013).

In light of these characteristics of weeds and their hazards, it becomes imperative to control them. Therefore, efforts are being made to find out alternative low input strategies for weed management, although numbers of management practices are available. Where allelopathy is, the direct influence of a chemicals released from one plant in the environment and then influence on the growth and development of another (Babula *et al*., 2009; Farooq *et al.*, 2011; Cheng and Cheng, 2015; El-Khatib *et al*., 2016). The allelopathic nature of about 240 weed species are reported and interfere with the growth and production of crops (Qasem and Foy, 2001; Singh *et al.*, 2003c). In this regard, allelopathic plants and their products for managing weeds in a sustainable manner has been focused with much attention (Sodaeizadeh *et al.*, 2010). Allelochemicals released from the allelopathic plants replace the use of synthetic herbicides for weed management and therefore, cause less pollution, safer agricultural products (Sodaeizadeh *et al.*, 2010) as well as alleviate human health concerns (Khanh *et al*., 2007). Suppressing weeds by harnessing the allelopathic phenomenon is included among the important innovative weed control methods (Jabran and Farooq, 2013; Zeng, 2014). Allelopathic weed control may be

applied as a single strategy in certain cropping systems, such as organic farming. Further, it can be combined with other methods to achieve integrated weed management. Under allelopathic weed control, the allelopathic potential of crops are manipulated in such a way that the allelochemicals from these crops reduce weed competition. The living plants or their dead materials express the allelopathic activity through the exudation of allelochemicals. Allelopathic weed control can be implemented by growing allelopathic plants in close proximity to weeds which promote production of these chemicals (Tesio and Ferrero, 2010) or by placing the allelopathic materials obtained from dead plants in close proximity to weeds. The decomposing plant material releases allelochemicals which are absorbed by the target weeds. The most important example for such cases includes the use of allelopathic plant residues for weed control (Tabaglio *et al.*, 2013). Allelopathic weed control can also be implemented by growing allelopathic plants in a field for a certain period of time, in order for their roots to exude allelochemicals. Crop rotation is the most important example for such allelopathic weed control (Farooq *et al*., 2011). Another way to control weeds through allelopathy includes obtaining allelochemicals in a liquid solution by dipping the allelopathic chaff in water for a certain period of time. Several researchers have advocated using this way of weed control either alone or in combination with other methods of weed control (Jabran *et al*., 2010; Khan *et al*., 2012; Razzaq *et al*., 2010, 2012). Therefore, for the management of agricultural weeds, it is worthwhile to explore the strong allelopathic activity of the plant.

In frequently disturbed areas, *Calotropis procera* (Ait.) R. Br. occurs as a major to intermediate weed and is found along the road verges throughout India (Sharma *et al*., 2010). Its widespread and persistent occurrence near barley, oat, rice, sorghum, maize, cotton, sugarcane fields and especially around wheat crop fields makes it suspicious to cause some adverse effect on these crops through allelopathic interaction (Yasin *et al*., 2012). Generally, the plant contains the allelochemicals that plays an important role in the formation of natural habitats and to compete with other species. For increasing organic materials in agroecosystems, it is recycled as a green manure, where it may change communities and inhibit crop growth and production (Al-Zahrani and Al-Robai, 2007). The successful invasion of *C. procera* can be attributed due to its continuous flowering and autogamy in invading areas, high seed production, efficiently dispersed by wind and fast growth after establishment (Sharma

et al., 2010; Leal *et al*., 2013; Sobrinho *et al*., 2013). In my allelopathic studies, aqueous and organic solvents, rhizosphere soil, residues and root residues of *C. procera* in various experiments, invariably reduced the germination, plumule growth, radicle growth, dry weight, carbohydrate content, chlorophyll content, protein content and caused alteration of chromosome morphology of their respective recipient species [weeds (*Cassia tora* L., *Cassia sophera* L., *Chenopodium album* L., *Cannabis sativa* L.) and crops (*Pisum sativum* L., *Triticum aestivum* L., *Brassica oleraceae* var. *botrytis*, *Spinacia oleracea* L. and *Allium cepa* L.)]. The study suggests that *C. procera* is allelopathic plant, which is capable of suppressing the germination and growth of various test species. Allelopathy is defined as the release of allelochemicals from the donor plant that affect the growth and development of receiver plants. Besides, various allelochemicals such as calotropin, catotoxin, calcilin and gigantin have been identified from *Calotropis* (Daubenmire, 1974; Kuriachen and Dave, 1989). The allelopathic nature of *C. procera* lead to the discovery of new products (allelochemicals) with their evaluation as an alternate strategy for biological control of other plant and organisms (Hirai, 2003; Bhowmik and Inderjit, 2003; Belz, 2007; Macias *et al*., 2007; Norton *et al*., 2008).

13.1. Rhizosphere soil of *C. procera* is phytotoxic in nature

The present study clearly indicated that the growth of various test plants was reduced in the rhizosphere soil. However, the percent emergence of test plant seeds was not affected, but there was a significant reduction in their growth. Both plant height and biomass of crops (*P. sativum*, *T. aestivum*, *B. oleracea* var. *botrytis*, *S. oleracea*) and weeds (*C. tora*, *C. sativa* , *C. sophera* and *C. album*) were reduced when grown in *Calotropis* invaded soil. However, the magnitude of inhibition varied from plant to plant. In general, maximum inhibition in root length and dry biomass was seen in *C. album* and in case of shoot length, it was seen in *C. tora*. Various recent studies (Batish *et al*., 2006a; 2007a; Sisodia and Siddiqui, 2009; Raoof and Siddiqui, 2012a; Fragasso *et al*., 2012; Iannucci *et al*., 2013; Safdar *et al*., 2014; Gulzar *et al*., 2011; Gulzar *et al*., 2014c; Gulzar and Siddiqui, 2015; Li *et al*., 2016) have evaluated the phytotoxic activity of rhizosphere soil that supports our finding. In *Calotropis* invaded soil, identification of four allelochemicals (ferulic acid, vanillic acid, *p*-coumaric acid and benzoic acid), along with the appreciable amount of phenolics was detected that might retard the growth of test plants. It is thus the

presence of phenolic acids in rhizosphere soil that might be reducing the growth of test plants as earlier reported (Fragasso *et al*., 2012; Iannucci *et al*., 2013; Liu *et al*., 2013; Liu *et al*., 2014). The possible role of soil nutrients was ruled out as none of the estimated nutrients was found to be deficient in the rhizosphere soil. Further, the pH and other properties of the soil also do not point any likely effect on growth of test plants

13.2. Phytotoxicity contributed by different parts of *C. procera*

It is clear from the present study that different parts of *C. procera* exhibited phytotoxic potential through their aqueous extracts, though the magnitude of phytotoxicity varied with plant part (i.e. root, stem and leaves). Not only the allelopathic activity of the weed, change with plant part but also with the concentration as noticed in the present study. Phytotoxic nature of aqueous extract of weed in nature that reduced the growth of other plants have been indicated by number of studies (Qasem and Foy, 2001; Bulut *et al*., 2006; Gulzar and Siddiqui, 2013b, Gulzar and Siddiqui, 2014a,b,c; Gulzar *et al*., 2014ab; Gulzar *et al*., 2015a; Gulzar and Siddiqui, 2016). The recent phytotoxic/allelopathic effect of aqueous extract of weeds include *Chenopodium album*, *Amaranthus retroflexus* and *Cynodon dactylon* (Rezaie and Yarnia, 2009), *Bothriochloa laguroides* var. *laguroides* (Scrivanti, 2010), *Amaranthus retroflexus*, *Chenopodium album*, *Erigeron canadensis* and *Solanum nigrum* (Marinov-Serafimov, 2010), *Achillea biebersteinii* Afan. (Abu-Romman, 2011), *Cassia tora* (Sarkar *et al*., 2012), *Chenopodium album* (Majeed *et al*., 2012), *Chenopodium murale* and *Malva parviflora* (Al-Johani *et al*., 2012), *Amaranthus hybridus* (Amini and Ghanepour, 2013), *Coronopus didymus* (Khaliq *et al*., 2013b), *Euphorbia guyoniana* (Nasrine *et al*., 2013), *Cannabis sativa* (Pudelko *et al*., 2014), *Trigonella foenum-graecum* (Omezzine *et al*., 2014a,b), A*geratum conyzoides* (Jayaraman and Ramalingam, 2014), *Cassia sophera* (Gulzar *et al*., 2014a), *Chrysanthemoids monilifera* ssp. *monilifera* (Al Harun *et al*., 2014), *Cleome arabica* and *Capparis spinosa* (Ladhari *et al*., 2014), *Cymbopogon nardus* (Suwitchayanon and Kato Noguchi, 2014) and *Salvia plebia (*Husna *et al.*, 2016). The observed phytotoxicity of *C. procera* may be attributed to the presence of variable amounts of phytotoxic substances in different parts and reduced the seeling growth and dry biomass of test species. The observed morphological changes such as seeds appear darkened and swollen, roots or radicles and shoots or coleoptiles reduced, root axes

curled, root hairs number reduced or increased, negative geotropism, increased number of seminal roots, reduced lateral root production, decreased root extension, root tips swelled (club-like appearance) or affected by necrosis in response to leaf aqueous extract in the present study corresponds well to other authors (Olson and McKercher, 1985; Chon *et al.,* 2002; Bhadoria, 2011; Pudelko *et al*., 2014). The SEM analysis of the leaf surface of *Cassia* revealed disruption of epidermal cells in the form of canals and formation of cyst like structures instead of being smooth as depicted in the control treatment. These observations are confirmed by a similar findings reporting epidermal cell morphology alteration in *Arabidiopsis thaliana* on exposure to monoterpenes allelochemicals viz; camphor and menthol (Schulz *et al*., 2007). Considering that the reduction in the germinability of test species was due specifically to the presence of substances with allelopathic activity in the extracts of *C. procera* that leach out in water solutions as the osmotic potential of -0.046 to -0.096 bars and pH of extracts ranged from 6.66 to 6.95 did not interfere with the germination of donor species. In our study, the foliar aqueous extract was noticed to be more allelopathic than the root and stem aqueous extract due to proportionally greater biomass and site of synthesis of chemicals.

13.3. Allelopathic interference of *C. procera* residue

C. procera grows abundantly especially throughout the year forming its own monocultures in agricultural fields and other ecosystems. Consequently, it produces large amount of residues under field conditions that also impart a significant phytotoxic nature to the weed**.** Seedling growth and dry biomass of test species *S. oleracea*, *B. oleracea* var. *botrytis*, *C. sativa* and *C. album* were found to be significantly reduced due to the phytotoxic nature of residue amended soil (RS), residue extract amended soil (RES) and residue extract (RE) compared to unamended soil (US). The test plants exhibited varying degrees of inhibition with maximum retardatory effect noticed in *C. album*. At the highest concentration of amended soils and extracts, maximum retardatory effect on all the test plants was observed. However, the magnitude of phytotoxicity by residue extract was more since the seeds were directly subjected to extracts in Petri dish bioassay. As per reports, even the growth and development of crop plants gets negatively impacted by the incorporation of residues from the invasive weeds (Batish *et al.* 2006a,b, 2007a,b; Batish *et al.,* 2009a). In our study, a significant high amount of phenolics was found to be present

in aqueous extracts of residue. In case of the weed *C. procera*, profuse growth along with its dominance in the infested area and continuous influx of plant residues maintains the availability of phytotoxic compounds into the soil. The dynamics of the release of phenolics in soil were, however, different compared to simple extracts. Phenolic content showed a periodic increase over time achieving peak (Khaliq *et al*., 2011) at their respective time and after which a decrease in phenolic content was observed in RS, RES and RE. This difference may be due to their relative release from the respective treatments. It is in agreement with earlier reports indicating that the composition and quantity of allelochemicals may vary substantially over the time or with changing environmental conditions (Wojcik-Wojtkowiak *et al.*, 1990; Blum, 1998; Khaliq *et al*., 2011). Rather, the residue amendment improved the nutrient status of the soil as also indicated by enhanced electrical conductivity and thus greater nutrient availability (Batish *et al*., 2009a). Soil pH was lowered in RS and RES compared to US. Since the phenolics make the soil acidic, such a lowering of soil pH is not surprising (Dalton *et al*., 1983; Batish *et al*., 2009a). Amount of organic matter and available nutrients was increased in the amended soil and the increase was more in RS than in RES as also revealed by Batish *et al*. (2009a). Therefore, the possibility of any resource depletion upon residue incorporation and their negative role in causing growth (Batish *et al*., 2009a) has been ruled out in the present study due to observed significant increase in the available nutrient content in RS and RES. The same results were reported from soil incorporation of residues from allelopathic plants by enriching the soil nutrient status rather than depriving it (Batish *et al*., 2002, 2007a; Batish *et al*., 2009a). Therefore, as per the study of Batish *et al*. (2009a), it indicated seedling growth inhibition might be due to direct involvement of the phenolics released from residue of *C. procera*.

13.4. Root residue mediated allelopathic interference of *C. procera*

The release of allelochemicals by root exerts allelopathic/phytotoxic effect on other plants is depicted from the present study. In order to establish this, aqueous extract from the root residue (RRE) of *C. procera* were prepared and *Brassica oleracea* var. *botrytis, S. oleracea, C. sativa* and *C. album* were tested to their phytotoxicity. Form the results, it is evident, that aqueous extracts of root reduced the initial growth of test plants (*S. oleracea, B. oleracea* var. *botrytis, C. sativa* and *C. album*) in terms of root length, shoot length and dry biomass. Maximum effect was

observed on *C. album* and *C. sativa* and tested plants exhibited a differential activity towards the extracts. This clearly shows that RRE possess growth inhibitors that are water-soluble. Therefore, the phytotoxic nature of root residue extract gets further strengthened when these were incorporated into the soil. Similar results were reported by (Batish *et al.* 2006a, 2007a; Batish *et al*., 2009b) by the negative effect of root exudates and residues of invasive weeds *Chenopodium album, Chenopodium murale* and *Ageratium conyzoides*, respectively. In growth studies, soil amended with aqueous extract of roots, induce the retardatory impact on growth of test plants (Javaid *et al.,* 2006; XiaoQing *et al.*, 2006). Batish *et al*. (2009b) stated that root exudates and root residues of *A. conyzoides* impose an inhibitory effect satisfying its allelopathic behavior is in line with our study. Very less change in soil pH was observed in RRS, RRES and RRE. However, with the amendment of residues at 2% and 4% the soil were more alkaline, i.e. pH increased towards alkalinity. In the present study, however, the analysis of the RRS and RRES indicated that availability of the nutrients is not a limiting factor and thus not a reason for the observed inhibitory effects is in agreement with the results of Batish *et al*. (2009b). The soils were nutrient rich, with an increased EC indicating greater nutrient availability and thus enrichment of the RRS and RRES. This is in line with the earlier reports where incorporation of residues or decomposing material of allelopathic plants into the soil can enhance nutrient status (Batish *et al*., 2002, 2007a; Batish *et al*., 2009b) and EC (Xuan *et al*., 2005; Batish *et al*., 2009b). Increase in the available soil nutrients, EC and pH of the soil has also been noticed upon amendation of decomposing residues of the invasive weed *Ageratium conyzoides* (Batish *et al*., 2009b), *Parthenium hysterophorus* (Batish *et al*., 2002) and *Chenopodium murale* (Batish *et al*., 2006a). In the present study, there was a substantial increase in the available N content as per study of Batish *et al*. (2009b). Moreover, phytotoxicity and quantification of phenolics from the RRS and RRES indicates their direct involvement in the observed growth reduction. Presence of significant amount of water soluble phenolics in the RRS and RRES indicated that these are primarily responsible for the observed growth reduction in these soils as has been previously investigated by Batish *et al*. (2009b). Besides, decomposing plant residues, including intact roots, water-soluble phenolics are the ubiquitous organic biomolecules released and widely implicated in allelopathic

interactions (Mizutani, 1999; Bertin *et al*., 2003: Xuan *et al*., 2005; Djurdjevic *et al*., 2008; Batish *et al*., 2009b).

13.5. Allelopathic impact on germination and physiological parameters by aqueous extract and organic solvents

Differential level of phytotoxicity in response to aqueous extracts and organic fractions was exhibited against test species (*T. aestivum, S. oleracea, C. album* and *C. sativa*) that might arise due to the variable chemical nature of the compounds used for extraction (Tanveer *et al*., 2012). Seeds of test species do not germinate in an environment that possesses allelochemicals from *C. procera* has been noticed from the results. The results are in consistent with the findings of (Tefera, 2002; Stavrianakou *et al.*, 2004, Dongre and Yadav, 2005; Kadioglue *et al*., 2005; Oyun, 2006; Siddiqui *et al*., 2009; Tanveer *et al*., 2010; Hussain *et al*., 2011; Ahmad, 2012; Novoa *et al*., 2012; Oluwole *et al*., 2013) who revealed suppression in the germination rate and final germination of target species by allelopathic plants. The phytotoxic effect of leaf leachates in aqueous and organic solvents was assessed on chlorophyll content, protein content and carbohydrate content. In the present study, as compared to the control, chlorophyll content in the test species decreased dramatically and the aqueous leachates proved to be much more effective than the other treatment in this case too. Several studies reported the decrease in chlorophyll contents with increase in concentration of allelopathic phenolics (vanillic acid, *o*-hydroxyphenyl acetic, *p*-hydroxybenzoic acid, ferulic and *p*-coumaric acids) in rice cabbage, Chinese fir, *Echinochloa crus-galli*, *Chenopodium album*, maize (Chen *et al*., 2002; Yang *et al*., 2004; Al-Sobhi *et al*., 2006; Jaleel *et al*., 2008; Sarkar *et al*., 2012; Singh *et al*., 2013) that coincides with our results. Similarly, the decrease in protein content in the test species followed the similar trend as chlorophyll content. The test species pronounced their inhibitory effect more in aqueous leachates than in organic solvents and control. Besides, it also varies from species to species. The findings in the present investigations are also in confirmation with these reports (Iman *et al*., 2006; Abu-Romman *et al*., 2012; Ibrahim *et al*., 2013, Gulzar and Siddiqui, 2014b) with decreased amount of protein in treated species while studying allelopathic effects. Reduction in rate of protein synthesis occurred by incorporation of certain aminoacids into proteins (Baziramakenga *et al*., 1997). One thing is very clear from the result of this experiment that *C. procera* leaves allelopathic exert a very negative influence on

the acid soluble and water soluble carbohydrates of test plants. It is very well depicted by an increased amount of carbohydrates content exerts its influence mainly through its aqueous leachates, i.e. in its glucosidic form. The results are in line with findings of (Sahar *et al*., 2005; Abdulghader *et al*., 2008; Gulzar and Siddiqui, 2014b) where appreciable increase in the increased concentration of soluble sugars occurred in response to various allelopathic agents.

Water is a polar compound while chloroform, petroleum ether, methanol is non-polar in nature. Tanveer *et al*. (2012) stated that different extraction efficiencies of the solvents lead to variable phytotoxicity of aqueous and organic fractions of *C. procera* accounting for qualitative and quantitative differences in extracted phytotoxins in different fractions. As shown by impaired germination, retarded seedling growth, decrease in chlorophyll and protein content and increase in carbohydrate content exhibited by aqueous leachates of *C. procera* reveals water as the best solvent to be used for extraction of inhibitory compounds (Tanveer *et al*. 2012). This is of great ecological significance in allelopathic interference on behalf of water-soluble compounds particularly in cultivated fields that are infested with this weed and are frequently irrigated or receive rainwater (Tanveer *et al*., 2012). As per study of (Khaliq *et al.*, 2011; Tanveer *et al*., 2012), the differential inhibition by allelopathic products is in agreement with our results. Moreover, sample preparation and extraction techniques is believed to lead variation in magnitude of allelopathic suppression (Zielinski and Kozlowski, 2000; Javaid *et al*., 2011)

13.6. Identification of allelochemicals and their phytotoxicity

From the various studies, the nature of allelochemicals was determined to be a group of heterogenous chemicals, basically comprised of phenolic acids, coumarins, alkaloids, flavonoids, etc. However, phenolic cids are the most common allelochemical group and also known to cause adverse effects on the other plants. In *C. procera*, phenolic acids namely caffeic acid, gentistic acid, catechol, gallic acid, syringic acid, ellagic acid, resorcinol, *p*-coumaric acid, *p*-hydroxy benzoic acid, vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, pyrogallic acid, furoic acid and ferulic acid were identified from leaf, stem and root of *C. procera.* The phenolics acids identified with different peak, retention time and quantities in different parts of the plant. In the green leaves, nine phenolic acids were detected. These included caffeic acid, gentistic acid, catechol, gallic acid, syringic cid, ellagic

acid, resorcinol, *p*-coumaric acid and *p*-hydroxy benzoic acid. Although eight phenolic acids (vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, syringic acid, gallic acid, pyrogallic acid and *p*-coumaric acid detected in stem and root extract were similar, however they show difference in terms of retention time and quantities that reflect their differential pytotoxicity. However, the furoic acid in stem extract and ferulic acid in root extract were identified different ones with their respective retention time and quantities in addition to these eight phenolic acids. These phenolic allelochemicals are known to exert allelopathic effect on the other plants. Phenolic acids are predominantly found in allelopathic plants and are synthesized within the plants as secondary metabolites. Within plant, remain in glycosidic form to avoid intra-plant toxicity and/ or facilitate movement within and outside the plant. In the last three decades, the potential of phenolic acids as allelochemicals has been widely described in the literature, not only in laboratory bioassays but also in field studies (Chung *et al*., 2002; Inderjit *et al*., 2002; Iqbal *et al*., 2003; Beninger *et al*., 2004; Djurdjevic *et al*., 2004; Sanchez-Moreiras *et al*., 2004; Kim *et al*., 2005; Blum and Gerig, 2005; Baratelli et al., 2012; Gulzar *et al*., 2015a; Ren *et al*., 2015; Liu *et al*., 2016). All these studies indicate that phenolic acids are one of the most common groups of allelochemicals found in a number of allelopathic plants. The reason for their wide spread occurrence could be their release from the donor plant through leachation as they can be easily solubilized in water. However, in our studies, only phenolic acids were identified since our emphasis was on water-soluble or leachable allelochemicals that accumulate in soil and impart phytotoxicity in it. Based on these observations, it could be concluded that phytotoxicity of *C. procera* could be attributed to a diversity of water soluble allelochemicals-phenolic acids.

13.7. Structures present on leaf surfaces contribute phytotoxicity

Scanning electron microscopy was used to determine the appearance of the leaf surface of the *C. procera*. It was found that the leaves of *C. procera* contain dense non glandular trichomes (NGTs) and stomata that are embedded into a thick cuticle on the surface of the leaves. Functionally, the forms and sizes of trichomes have been linked to the accumulation and secretion of organic metabolites on the epidermal layers of studied tissues. In this study, the presence of anisocytic stomata and filamentous non glandular trichomes (NGTs) revealed by the SEM were the major ultrastructures on the upper (adaxial) and lower (abaxial) leaf surfaces of *C. procera*

that contribute the allelopathic activity. These observations are supported by similar findings earlier reported by (Adedeji and Jewola, 2008; Dixon *et al*., 2008; Badmus and Afolayan, 2012). Dipping experiment conducted to determine whether the allelochemicals contained in trichomes are organically soluble or insoluble using dichloromethane. It was found in this experiment that the alleochemicals in trichomes on the leaves of *C. procera* are not organically soluble, as the effect of dichloromethane infusion did not significantly affect the seedling growth of *T. aestivum* as compared to distilled water infusion. This confirms that structures present on leaf surfaces contain the inhibitory substances (allelochemicals) that are water soluble. Secondary metabolites sequestered in glandular trichomes of many plants exhibit their phytotoxicity on seed germination and seedling growth of other plants earlier revealed by the authors (Oyedeji *et al*., 2005; Sultana and Afolayan, 2007; Dixon *et al*., 2008). The occurrence of trichomes on the leaf surfaces of C. *procera* and secretion of their contents might also be responsible for allelopathic behavior of *C. procera*. Structures found on the leaves of the plant could possibly contain the allelochemicals used by the plant to ensure its successful invasion growth. This also supports the findings that leaves being more in biomass per plant contributed relatively more towards phytotoxicity compared to other parts of the plant.

13.8. Leaf aqueous extract induces cytomorphological changes

The results of cytological changes with particular reference to significant alteration of mitotic cell division behavior and chromosomal anomaly because of treatment of root tip cells of *A. cepa* with the leaf aqueous extract of *C. procera* were represented. The alteration in mitotic stages were noticed in prophase, metaphase, anaphase and telophase particularly. The inhibitory effect was marked in all treatments (0.5,1%,2% and 4%) of leaf aqueous extract in the root tip cells of *A. cepa* compared to control sample. Whereas, at the 0.5% concentration, the alteration in anaphase stage was not detected. Further, the telophase stages remain unaffected at 0.5%, 1% and 2% concentration of leaf aqueous extract. Decrease in the mitotic index of this study clearly indicates the cytotoxic effect and such reports are available in the literature (Ukaebu and Odeigah, 2009). The allelochemicals present in aqueous extract of *C. procera* such as phenolic compound might be responsible for cytotoxicity in *Allium* root tip cells supporting the above view. Similarly, leaf aqueous extract are also a source of different types of allelochemicals with their potent

inhibitory/retardant activities on different enzymes, hormones and nucleic acids involved in cell division. Our results are in consistent with cytotoxic effects recorded earlier (Padhy *et al*., 2006; Priyadarshani, 2006; Prasad and Priyadarshani, 2006; Sreelaraj *et al*., 2007; Sousa *et al*., 2007; Dragoeva *et al*., 2008).

Various types of chromosomal abnormalities (stickiness, delayed mitosis, distrubed phases, micronuclear formation, bridges, lagging chromosones, C and U mitosis) were induced upon treatment of leaf aqueous. Chromosomal aberrations may be due to the nucleotoxic action of extracts or the disturbance of the formation of spindle fibres during cell division (Nwakanma and Okoli, 2010). Treatment of root tips with the leaf extract of *C. procera* usually experienced with common forms of chromosome abnormalities such as stickiness, distrubance and clumping. Such types of anomaly were known to occur in cells treated with spindle inhibitor and antimitotic chemicals (Bazer *et al*., 1975; Deyson, 1975). The shortening and constriction of the chromosomes and condensation of daughter nuclei in treated samples revealed the impairment of mitotic activity by the leaf extracts. Stickiness and clumping of chromosomes noticed in treated cells revealed the depolymerisation effects of the leaf extracts on the nucleic acids of the chromosomes. Higher percentage of abnormalities was recorded in cells treated with higher concentration (4%) of leaf aqueous extract. The data presented here indicate that leaf aqueous extract of *C. procera* have the potentiality of inducing a variety of abnormalities in the root tip cells of *Allium*.

Conclusion

CONCLUSION

- Rhizosphere soil of C*alotropis procera* (Ait.) R. Br. significantly affects the growth of test species by releasing water soluble phenolic allelochemicals. Therefore, the possibility of any nutrient depletion in causing negative growth has been ruled out.
- Different parts of *C. procera* exhibit differential phytotoxicity and the degree of phytotoxicity with respect to plant part was in the order: Leaves > Roots > Stems. Leaves are more in biomass per plant contributed relatively more towards phytotoxicity compared to other parts of the plant. Presence of phenolics imparted the allelopathic/ phytotoxic property to the different parts as evidenced from their amount and degree of inhibition of test plants.
- Various applications of *Calotropis* residues (RS, RES and RE) can be successfully used in weed control management. The residue of *C. procera,* like its fresh parts was allelopathic in nature causing a significant retardatory effect on the crops and weeds by releasing water soluble phenolic acids into the soil environment and not through depletion of available soil nutrients. Use of *Calotropis* as allelopathic agent will be a new but eco-friendly, cheaper and effective mode of weed control.
- The allelopathic effect of *Calotropis* root in terms of RRS, RRES and RRE on seedling growth and dry biomass is mainly attributed due to the detection of phenolics indicating that the availability of the nutrients is not a limiting factor and thus not a reason for the observed inhibitory effects.
- Differential level of phytotoxicity in response to aqueous leachates and organic fractions on germination parameters and physiological parameters was exhibited against test species that might arise due to the variable chemical nature of the compounds used for extraction. Further, different extraction efficiencies of the solvents, lead to variable phytotoxicity of different aqueous and organic fractions of *C. procera* accounting for qualitative and quantitative differences in extracted phytotoxins in different fractions.
- The whole allelopathic impact of *C. procera* is mainly contributed by phenolic acids identified from leaf, stem and root such as caffeic acid, gentistic acid, catechol, gallic acid, syringic acid, ellagic acid, resorcinol, *p*-coumaric acid, *p*-hydroxy benzoic acid, vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, pyrogallic acid, furoic acid and ferulic acid. It can be concluded that the

identified chemicals might have rendered the adverse effect on growth behavior, physiological, biochemical, cytological and anatomical parameters during this investigation.

- The results of the current study have demonstrated that *C. procera* possess some ultrastructures (trichomes) which might be functionally responsible for the production and storage of allelochemicals such as phenolic acids. Results concurred that the allelochemicals causing allelopathic potential of *C. procera* are probably water-soluble (polar compounds). Allelopathic repercussions could happen once the plant has died and decaying matter in the grass field becomes wet in rainy seasons.
- Leaf aqueous extract exhibit pronounced effect on the mitotic activity of *A. cepa* which may be due to their rich phytochemistry. The *Allium* test for cytogenetic studies allows for a more detailed insight into the modes of allelopathic action.

Summary

SUMMARY

Calotropis procera (Ait.) R. Br. commonly known as 'Aakawa' belonging to the family Asclepiadaceae, is an erect perennial shrub whose members are distributed throughout the world in tropical and sub-tropical regions. With wide ecological distribution in many regions of Aligarh district of Uttar Pradesh, India, it mainly shows its common occurrence and invasion around the agricultural lands and farms. In frequently disturbed areas, it occurs as a major to intermediate weed and throughout India, it shows its presence along along roadsides, streets, residential colony parks, sand dunes as well as in crop fields as a weed. Generally, the plant contains the allelochemicals that plays an important role in the formation of natural habitats and to compete with other species. The dominance and establishment of *C. procera* can be contributed due to its flower that bloom contineously and fertilization of a flower by its own pollen, production of large number of seeds and their dispersion by anemophily and fast growth after establishment. Due to the allelopathic nature of *C. procera* reported by several authors and the fact that the plant is invading at an alarming rate in cultivated and agricultural lands thereof, a study of the plant's allelopathic potential was undertaken in Aligarh district. Allelopathy is a form of interference competition where the allelochemicals released from the donor plant affect the growth and development of receiver plants.

Preliminary experiments were performed to determine phytotoxicity of rhizosphere soil on seedling growth and dry biomass of test species, the potential of which varied from species to species. The growth of test species (*Pisum sativum* L., *Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., *Spinacia oleracea* L., *Cassia sophera* L., *Cassia tora* L., *Cannabis sativa* L. and *Chenopodium album* L.) when grown in *C. procera* infested soil was significantly affected compared to control as clearly depicted from the experiments. Both test plants (crops/weed) height and biomass accumulations were significantly reduced in *C. procera* invaded field soil. Generally, in *C. album* a greater retardatory effect was seen as compared to other species. On the basis of root length of the test plants, the decreasing order of sensitivity appeared to be *T. aestivum>P. sativum>S. oleracea>B. oleracea* var. *botrytis>C. sophera>C. tora>C. sativa>C. album.* On the basis of shoot length, the decreasing order of test plants was shown to be *T. aestivum>P. sativum>S. oleracea>B. oleracea* var. *botrytis>C. sophera>C. album>C. sativa>C. tora.* In the case of dry weight, the decreasing order of sensitivity of the test plants followed the

trend *T. aestivum>P. sativum>S. oleracea>B. oleracea* var. *botrytis>C. sophera>C. sativa>C. tora>C. album.* Not much change in soil pH was observed between C. *procera* invaded site and the control soil. The pH was slightly alkaline or even near neutral. The electrical conductivity, however, was more in the soil collected from *C. procera* infested soil. The percent organic carbon and organic matter found to be maximum in soil supporting *C. procera* plants compared to control soil. Further, amounts of all the nutrients (whether macro-or micro-or ions) was more in *C. procera* field soil compared to control soil and hence they are not responsible for growth retardatory effects of crops. In *Calotropis* invaded soil, identification of four allelochemicals (ferulic acid, vanillic acid, *p*-coumaric acid and benzoic acid), along with the appreciable amount of phenolics was detected. The presence of phenolics might be adversely affecting the growth of plants grown in rhizosphere soil.

The aqueous extract prepared from leaves, stem and root of *C. procera* with its allelopathic impact were performed to determine their phytotoxicity. Crop plants, i.e. *T. aestivum, B. oleracea* var. *botrytis, S. oleracea, P. sativum* and weed plants, i.e. *C. sophera, C. tora, C. album* and *C. sativa* was used as test species. Root length, shoot length and dry biomass were reduced when exposed to aqueous extract of respective parts. The toxicity of plant extracts was concentration dependent, thus an increase in retardatory activity of extracts was observed with their increasing concentrations. Considering that the reduction in the germination of test species was due specifically to the presence of substances with allelopathic activity in the extracts of *C. procera* that leach out in water solutions as the osmotic potential of -0.046 to -0.096 bars and pH of extracts ranged from 6.66 to 6.95 did not interfere with the germination of donor species. Further, the morphological alteration in seedlings has been induced in *P. sativum* and *C. sophera* when exposed to leaf aqueous extract. The observed morphological changes such as seeds appear darkened and swollen, roots or radicles and shoots or coleoptiles reduced, root axes curled, root hairs number reduced or increased, negative geotropism, increased number of seminal roots, reduced lateral root production, decreased root extension, root tips swelled (club-like appearance) or affected by necrosis in response to leaf aqueous extract. The SEM analysis of the leaf surface of *Cassia* revealed disruption of epidermal cells in the form of canals and formation of cyst like structures instead of being smooth as depicted in the control treatment. In this study, the leaf aqueous extract exert a greater allelopathic impact followed by root and stem extract.

The allelopathic potential of residue amended soil (RS), residue extract amended soil (RES) and residue extract (RE) on seedling growth and dry biomass of test species (*S. oleracea, B. oleracea* var. *botrytis, C. sativa* and *C. album*) were investigated. The seedling growth was significantly reduced upon exposure to three types of treatment compared to unamended control (US). The test plants exhibited varying degrees of inhibition with maximum retardatory effect noticed in *C. album*. At the highest concentration of extracts and residues amended in soil, the maximum retardatory effect on all the test plants was observed. However, the impact on growth and dry biomass of test species were more in RE than in RS and RES as the seeds were directly exposed to extracts in Petri dishes. Near neutral pH of residue extract excludes its role in growth reduction. Soil pH was lowered in RS and RES compared to US. Amount of organic matter and available nutrients was increased in the amended soil and the increase was more in RS than in the RES. Upon analysis of the physicochemical characteristics of amended soil (RS and RES), the soils were nutritionally rich as compared to unamended soil (US). The nutritional contents were more in RS than in RES. Further, the presence of phenolics in RS, RES and RE increases with the increasing concentration of residue amended in soil and residue extract. This point to the fact that phenolics might be responsible for the growth retardatory effect and rules out the role of nutrient deficient soil in causing growth reduction.

The study was conducted with a view to understand the phytotoxic effect of its roots towards some crop and weed plants vis-à-vis interaction of their allelochemicals with soil properties. The root residue amended soil (RRS), root residue extract amended soil (RRES) and root residue extract (RRE) exhibit variability in their retardatory effect on seedling growth and dry weight of test species (*S. oleracea, B. oleracea* var. *botrytus, Cannabis sativa* and *C. album*) as compared to their control. The retardatory effect noticed was more in RRE than in RRS and RRES as the seeds of test species are in direct contact with extracts in Petri dishes. Maximum effect was observed on weed species (*C. album* and *C. sativa*). Very less change in soil pH was observed. However, with the amendment of residues at 2 and 4% and in extract, the soil were more alkaline, i.e. pH increased towards alkalinity and thus rules out its role in growth inhibition. RRS and RRES was found to be nutrient rich with increased EC and could not be the reason for the reduction in growth of test species as depicted in the present study. However, the detection of a significant amount of water soluble

phenolics in the amended soils (RRS and RRES) suggested their direct involment in the observed growth reduction.

The allelopathic impact of allelochemicals extracted from leaves of *C. procera* using aqueous leachates and organic fractions (petroleum ether fraction, methanolic fraction, chloroform fraction and water fraction**)** on physiological parameters was assessed. Chlorophyll content, protein content and carbohydrate content constitute the physiological parameters studied. The study was undertaken on four test species (*T. aestivum, S. oleracea, C. album* and *C. sativa*). As compared to the control, chlorophyll content in both the test species (crop and weed) decreased dramatically and the aqueous leachates proved to be much more effective than the other treatment. One thing is very clear from the result of this experiment that *C. procera* leaves allelopathic exert a very negative influence on the acid soluble and water soluble carbohydrates of test species. It is very well depicted by an increased amount of carbohydrate content exerts its influence mainly through its aqueous leachates, i.e. in its glucosidic form. The weed plants show a better sensitivity as compared to crop plants. An increased amount of carbohydrates points out to the fact that the plant is under stress and it is gathering up its energy reserves to meet any conditions of adversity. Further, it was observed that the plant protein content was found to be reduced in all the treatments as compared to control.

The experiment conducted for the extraction of allelochemicals from the leaves of *C. procera* in aqueous and organic solvents (petroleum ether fraction, methanolic fraction, chloroform fraction and water fraction**)** and to investigate their allelopathic behavior on germination parameters. The germination percentage, radical length, plumule length and seed vigour were studied on four test species (*T. aestivum, S. oleracea, C. album* and *C. sativa*). Germination dynamics and seedling growth of test species gets significantly inhibited upon analysis and interpretation of the results upon exposure to aqueous and organic fraction solvents. Seeds of *T. aestivum, S. oleracea, C. album, C. sativa* do not germinate in an environment that possesses allelochemicals from *C. procera* has been noticed from the results of this experiment. In fact, the inhibitory effect impacted by aqueous extracts on seedling growth was found to be much greater than was achieved with the organic fractions. Differential level of phytotoxicity in response to aqueous extracts and organic fractions exhibited against test species that might arise due to the difference in the chemical nature of the compounds used for extraction. Water is a polar compound while chloroform,

petroleum ether and methanol, is non-polar in nature. Qualitative and quantitative differences in identified allelochemicals in different solvents are due to the difference in extraction efficiencies of solvents that leads to differential allelotoxicity. The study depicted water as the best solvents for the extraction of phytotoxins as is evident by the impaired germination and seedling growth reduction in recipient species.

By advanced HPLC chromatographic analysis, identification of phenolic acids from leaves, stem and root of *C. procera* were revealed. The phenolic compounds vary in their retention time, peak height, area and quantities in different parts of the plant. This vital part of experiment clearly confirmed the existence of the allelopathic potential of the plant and the allelopathic action was triggered by a number of identified allelochemicals. In the green leaves, nine phenolic acids were detected. These included caffeic acid, gentistic acid, catechol, gallic acid, syringic cid, ellagic acid, resorcinol, *p*-coumaric acid and *p*-hydroxy benzoic acid. Although eight phenolic acids (vanillic acid, chlorogenic acid, protocatecheic acid, quercetin, syringic acid, gallic acid, pyrogallic acid and *p*-coumaric acid) detected in stem and root extract were similar, however they show difference in terms of retention time and quantities that reflect their differential pytotoxicity. However, the furoic acid in stem extract and ferulic acid in root extract were identified different ones with their respective retention time and quantities in addition to these eight phenolic acids.

Scanning electron microscopy was used to determine the appearance of the leaf surface of the *Calotropis* weed. It was found that leaves of *C. procera* contain the numerous non-glandular trichomes (NGTs) and stomata that are embedded into a thick cuticle on the surface of the leaves. In leaf dipping experiment using dicholoromethane and distilled water to determine the solubility of contents contained in the trichomes and their contribution on seedling growth inhibition of *T. aestivum.* used as test specie. From the results, the root length and shoot length was reduced significantly by distilled water infusions in contrast to dichloromethane infusions. It was found in this experiment that the content (allelochemicals) in the trichomes on the leaves of *C. procera* are water soluble. The findings strongly suggest that the main site of allelochemicals contained by the plant can be found on the leaves.

The allelopathic influence of a leaf aqueous extract of *C. procera* at the cytomorphology was investigated, their cytotoxicity was screened using *Allium cepa* L. as a test specie. The results indicated a reduction in mitotic index and relative division rate, however, there was a significant increase in relative abnormality rate.

The cytological anomalies recorded were also dose/concentration dependent. The phenolic allelochemicals detected in different parts might be inducing the chromosomal abnormalities. This has confirmed that the allelochemicals present in the rhizosphere soil, extracts and residues induced changes not only in germination parameters, seedling growth, morphology and physiology but they also induced changes in treated plants right from the cell and chromosome level which finally manifested at maturity level.